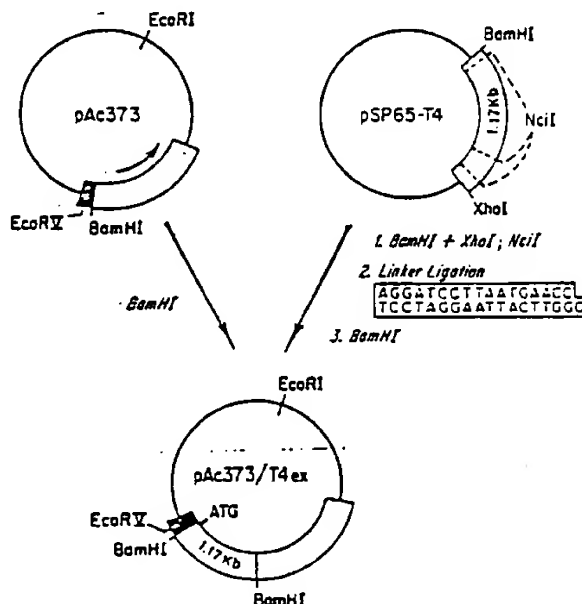




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(54) Title: SOLUBLE HUMAN CD4 FRAGMENTS AND USES THEREFOR



(57) Abstract

Modified soluble human CD4 fragments which are capable of binding HIV gp120 envelope protein and which do not interfere with the function and proliferation of T lymphocytes not infected by HIV, DNA encoding such fragments and uses therefor. Such fragments can be used for diagnostic, therapeutic and preventive purposes.

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SOLUBLE HUMAN CD4 FRAGMENTS AND USES THEREFORDescriptionBackground

The CD4 (T4) molecule, which is a surface glycoprotein on a subset of T lymphocytes (referred to as T4 lymphocytes) is involved in Class II (Ia) MHC recognition and appears to be the physiological receptor for one or more monomorphic regions of class II MHC. Meur, S. et al., Proceedings of the National Academy of Sciences, U.S.A., 79:4395-4399 (1982); Biddison, W. et al., J. Exp. Med., 156:1065-1076 (1982); Gay, D. et al., Nature, 328:626-629 (1987).

Human CD4 is also the receptor for the gp120 envelope glycoprotein of the human immunodeficiency virus (HIV) and is essential for virus entry into the host cell, and for membrane fusion, which both contribute to cell-to-cell transmission of the virus and to its cytopathic effects. Klatzmann, D., et al., Science, 225: 59-63 (1984); Dalglish, A.G., et al., Nature, 312: 763-766 (1984); Sattentau, Q., et al., Science, 234: 1120-1123 (1986); McDougal, J.S., et al., J. Immunol., 137: 2937-2944 (1986); McDougal, J.S., et al., Science, 231: 382-385 (1986); Maddon, P.J., et al., Cell, 47: 333-348 (1986); Sodroski, J., et al., Nature, 322: 470-474 (1986); Lifson, J., et al., Nature, 323: 725-728 (1986). Sequence analysis of CD4 has suggested an evolutionary origin from a structure with four immunoglobulin-related domains.

Clark, S., et al., Proc. Natl. Acad. Sci., 84: 1649-1653 (1987); Littman, D.R., et al., Nature, 325:

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453-455 (1987). Only the two NH<sub>2</sub>-terminal domains are required to mediate HIV gp120 binding. Trauneker, A., et al., Nature, 331: 84-86 (1988); Berger, E.A., et al., Proc. Natl. Acad. Sci. USA, 85: 2357-2361 (1988); Richardson, N.E., et al., Proc. Natl. Acad. Sci. USA, in press.

Considerable effort has been expended in studying the CD4-gp120 interaction and in trying to interfere with or inhibit that interaction, in an attempt to provide a means by which the life threatening effects of HIV infection can be slowed or reversed. Several groups have focused their efforts on the ability of soluble CD4 (T4) protein to interfere with infection of cells by HIV and its subsequent effects. Hussey, R.E. et al., Nature, 331:78-81 (1988); Fisher, R.A. et al., Nature, 331:76-78 (1988); Deen, K.C. et al., Nature, 331:82-84 (1988); Trauneker, A. et al., Nature, 331:84-86 (1988). A means by which to prevent HIV infection of T4 lymphocytes (i.e., helper and inducer T lymphocytes), which make up approximately 60-80% of the total circulating T lymphocyte population, would be of great value, particularly in light of the fact that HIV infection of such cells can cause total collapse of the immune system. Curran, J. et al., Science, 229:1352-1357 (1985); Weiss, R. et al., Nature, 324:572-575 (1986).

#### Disclosure of the Invention

The present invention relates to soluble human CD4 (T4) fragments which bind to the HIV gp120 envelope protein (HIV gp120); to soluble human CD4 fragments whose ability to bind to the HIV gp120

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envelope protein has been altered; to DNA encoding such types of human CD4 fragments; to methods of using soluble human CD4 fragments in interfering with infection of cells by HIV; to methods of modifying  
05 the amino acid sequence of soluble human CD4 fragments; and to methods of modifying or altering the ability of soluble human CD4 fragment to bind HIV gp120. (CD4 and T4 are used herein interchangeably).

Soluble human CD4 fragments include none of the  
10 hydrophobic transmembrane region of CD4 or only a portion (generally six amino acids or less) of the hydrophobic region which does not prevent solubilization of the fragments. As a general class or category, soluble human CD4 fragments which are  
15 capable of binding with HIV gp120 are referred to as biologically active soluble human CD4 fragments. As explained below, biologically active soluble human CD4 fragments can be modified, with the result that the amino acid sequence differs in some way from that  
20 of the corresponding portion of naturally-occurring CD4. All such fragments (i.e., those which correspond in amino acid sequence with the naturally-occurring CD4 and those which are modified) which are capable of binding HIV gp120 are included  
25 within the term biologically active soluble human CD4 fragments, as used herein. However, for ease of discussion, soluble CD4 fragments which have in some way been modified as to amino acid sequence are referred to as biologically active, modified soluble  
30 CD4 fragments. Those fragments whose HIV gp120 binding ability has been changed are referred to as modified soluble CD4 fragments with altered HIV gp120 binding ability. In those cases in which HIV gp120

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binding ability is increased or enhanced, such fragments can be referred to as biologically active, modified soluble CD4 fragments with enhanced HIV gp120 binding ability. Conversely, those whose  
05 binding ability is reduced can be referred to as modified soluble CD4 fragments with reduced HIV gp120 binding ability.

Biologically active soluble human CD4 fragments can be modified in several different ways. The amino  
10 acid sequence of soluble human CD4 can be:  
1) truncated; 2) altered by means of substitution(s) in, deletion(s) from and/or addition(s) to the amino acid sequence; or 3) both truncated and altered. These three types or classes of fragments can be  
15 referred to, respectively, as truncated, altered, and truncated/altered.

Biologically active soluble CD4 fragments of the present invention have the ability to bind to HIV. They will, therefore, also have the capacity to  
20 prevent infection of human T-lymphocytes by HIV and to prevent formation of the human T-lymphocyte syncytia which are thought to play a role in transmission of HIV from cell to cell.

Such biologically active soluble CD4 fragments  
25 can be used for diagnostic, therapeutic and preventive purposes. For example, they can be used to determine the presence or absence of HIV gp120 in a biological sample (e.g., blood, urine, saliva, semen) and, thus, to determine whether HIV is present  
30 in the sample or not. In addition, they can be used to treat individuals infected with HIV, in vivo (e.g., by administration to infected individuals). They can also be used prophylactically. That is,

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they can be administered to individuals at risk for HIV infection. Further, they can be used to prevent infection by HIV by, for example, being coated onto materials used as barriers against introduction of the virus (e.g., condoms, spermicides, garments, containers for collecting, processing or storing blood, etc.).

Modified soluble CD4 fragments with altered HIV gp120 binding ability can be used for diagnostic, therapeutic and preventive purposes. They can be used in a similar manner as described above for use of biologically active soluble human CD4 fragments.

#### Brief Description of the Drawings

Figure 1 is the nucleotide sequence of T4 SEC1 cDNA (referred to as the T4<sub>ex1</sub> sequence), which encodes 370 amino acids of soluble CD4 protein (referred to as T4<sub>ex1</sub>). Modifications in cDNA and in the encoded CD4 protein are indicated by the boxed areas; each box represents the nucleotide triplet and encoded amino acid at which the modification is made.

Figure 2 is a schematic representation of the construction of an expression vector of the present invention.

Figure 3 is a schematic representation of the method by which the biologically active, modified soluble CD4 fragments of the present invention are produced.

Figure 4 is a graphic representation of the effects of the soluble CD4 fragments of the invention

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and the effects of control proteins on viral protein replication.

Figure 5 is a graph illustrating the lack of inhibition of CTL effector function by the soluble  
05 CD4 fragments of the invention.

Figure 6 is a bar graph illustrating the lack of inhibition of proliferation of normal helper T-lymphocytes by the soluble CD4 fragments of the invention.

10 Figure 7 is a schematic representation of the structure of native and recombinant CD4 proteins. Figure 7A is a representation of the native CD4 protein structure derived from the cDNA sequence of Figure 1. Numbers in parentheses indicate the four  
15 putative extracellular domains; the S at 16, 84, 130, 159, 303 and 345 indicates the position of cysteine residues; Tm: transmembrane; Cty: cytoplasmic region. Figure 7B is a schematic representation of the T4<sub>ex1</sub> protein. Figure 7C is the complete amino  
20 acid sequence of the T4<sub>ex1</sub> protein.

Figure 8 is a schematic representation of CD4 protein T4<sub>ex1</sub> showing the four immunoglobulin-like domains, three disulfide bonds and two potential glycosylation sites. Numbering of amino acids is  
25 according to Hussey *et al.*, *Nature*, 331:78-81 (1988). The positions of 16 mutations (see the Table) are represented below the line. The triangle indicates a stop codon introduced by site directed mutagenesis to create a protein containing only the first 182 amino  
30 acids.

Figure 9 shows results of anti-CD4 immunoprecipitation and anti-gp120 co-precipitation of T4<sub>ex1</sub> and a truncated 182 amino acid version of



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CD4 from supernatants of <sup>35</sup>S-cysteine labelled Cos-1 cells transfected with the CD4 constructs. Lane 1, immunoprecipitation of supernatant from Cos-1 cells transfected with the T4<sub>ex1</sub> containing plasmid and immunoprecipitated with anti-T8 (21Thy2D3) (control); lane 2, immunoprecipitation of supernatant from Cos-1 cells transfected with the 182 amino acid truncation using the control anti-T8 antibody; lane 3, immunoprecipitation of T4<sub>ex1</sub> with anti-CD4 antibody (19Thy5D7); lane 4, immunoprecipitation of the 182 amino acid truncation of T4<sub>ex1</sub> with anti-CD4; lane 5, co-precipitation of T4<sub>ex1</sub> with anti-gp120 (DuPont) in the presence of gp120; lane 6, co-precipitation of T4<sub>ex1</sub> with anti-gp120 in the absence of gp120; lane 7, co-precipitation of the 182 amino acid truncation of T4<sub>ex1</sub> with anti-gp120 in the presence of gp120. All samples are run non-reduced. The molecular weight markers are phosphorylase B (97.4KD), bovine serum albumin (69KD), ovalbumin (46KD), carbonic anhydrase (30KD), lactoglobulin A (18.4KD).

Figure 10 shows results of anti-CD4 immunoprecipitation of <sup>35</sup>S-cysteine labelled supernatants from Cos-1 cells transfected with T4<sub>ex1</sub>, M5, M10, M7 and M3. Precipitations were carried out in the presence (+) or absence (-) of gp120.

#### Detailed Description of the Invention

The present invention relates to soluble human CD4 fragments which bind to HIV gp120, as well as to soluble human CD4 fragments having altered gp120 binding ability; to DNA encoding soluble human CD4 fragments; to methods of making soluble human CD4 fragments and to methods of using soluble human CD4

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fragments of the present invention to interfere with HIV infection of cells. In particular, it relates to soluble human CD4 fragments in which the amino acid sequence is the same as that of the corresponding region of naturally-occurring human CD4; to soluble human CD4 fragments in which the amino acid sequence has been modified, with the result that their amino acid sequences differ, as described below, from that of the corresponding region of naturally-occurring human CD4; to soluble human CD4 fragments whose binding ability is different from that of naturally-occurring human CD4 or the corresponding human CD4 fragment and to DNA encoding such soluble human CD4 fragments.

15 Soluble human CD4 fragments of the present invention include none of the hydrophobic transmembrane region of naturally-occurring CD4 or contain a portion of the hydrophobic region which is sufficiently short (i.e., generally six amino acids or less) that it does not prevent solubilization of the fragments. Soluble human CD4 fragments capable of binding HIV gp120 are referred to herein as biologically active soluble human CD4 fragments. Biologically active soluble human CD4 fragments are 25 long enough (e.g., 10 amino acids or longer) that they are able to bind effectively to HIV gp120. Fragments need not exhibit total homology with the amino acid sequence of the corresponding region of human CD4. Rather, they must have sufficient 30 homology to bind to HIV gp120.

In addition, biologically active soluble human CD4 fragments of the present invention are able to exert an anti-HIV effect, as a result of binding HIV

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gpl20, without interfering with the function or proliferation of human T-lymphocytes not infected with HIV. That is, biologically active soluble human CD4 fragments of the present invention have been  
05 shown, as described below, to prevent infection of human T-lymphocytes by HIV and to inhibit HIV envelope-induced syncytium formation and HIV replication without inhibiting Class II MHC recognition events (i.e., without inhibiting CTL  
10 effector function), even at high concentrations, and without having a discernible effect on Class II-directed physiologic T cell responses.

As used herein, the term soluble human CD4 fragments includes all soluble human CD4 fragments  
15 (i.e., those in which the amino acid sequence corresponds to that of naturally-occurring human CD4 and those in which modification of amino acid sequence has been made) capable of binding HIV gp120. Biologically active soluble human CD4 fragments in  
20 which the amino acid sequence has been modified are referred to biologically active, modified soluble human CD4 fragments. Fragments whose HIV gp120 binding ability has been changed (with the result that it is different from that of the corresponding  
25 or equivalent portion of naturally-occurring CD4) are referred to as modified soluble human CD4 fragments with altered HIV gp120 binding ability.

Biologically active, modified soluble human CD4 fragments of the present invention differ from that  
30 of soluble human CD4 (e.g., from the sequence represented in Figures 1 or 7C) in that the amino acid sequence: 1) is truncated; 2) has been altered as a result of deletion(s) from, substitution(s) in

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and/or addition(s) to the amino acid sequence of human CD4; or 3) it is truncated and the truncated form or portion includes deletion(s) from, substitution(s) in and/or addition(s) to the amino acid sequence which occurs in the corresponding portion or segment.

Modified soluble human CD4 fragments having altered HIV gp120 binding ability are modified soluble human CD4 fragments in which the amino acid sequence of soluble human CD4 is altered at a selected site or sites in such a manner that the resulting CD4 fragment has HIV gp120 binding ability or affinity less than that of the corresponding (unaltered) soluble human CD4 fragment, or HIV gp120 binding ability or affinity greater than that of the corresponding (unaltered) human CD4 fragment. Such fragments are referred to, respectively, as modified soluble human CD4 fragments with diminished HIV gp120 binding ability and modified soluble human CD4 fragments with enhanced HIV gp120 binding ability.

In particular, CD4 fragments with altered HIV gp120 binding ability differ from soluble human CD4 fragments in that the amino acid sequences of the CD4 fragments with altered gp120 binding ability are different from the amino acid sequence of the soluble CD4 protein at a site or sites which have been found to be critical for gp120 binding. Until the present time, it has not been possible to selectively alter gp120 binding ability of soluble CD4 fragments because sites critical to gp120 binding had not been identified. Such critical sites have now been identified by means of oligonucleotide-directed mutagenesis and have been found to occur in domain I

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and domain II of human CD4 protein, suggesting that the HIV gp120 binding site is complex and involves both of the NH<sub>2</sub>-terminal domains. Modifications of the T4 cDNA, as it is represented in Figure 1, have been made and the encoded CD4 fragments expressed. Resulting CD4 fragments have been shown to have altered gp120 binding ability in vitro; in these instances, gp120 binding ability has been abrogated. Modifications at these same sites, and at other, as yet unidentified, sites, as described herein, can similarly be made to enhance gp120 binding ability, as well as to reduce or turn down (but not eliminate) gp120 binding ability.

The following is a brief description of the methods by which soluble human CD4 fragments of the present invention were produced; these are subsequently described in detail in the Examples.

#### Production of Biologically Active Soluble Human CD4 Fragments

Construction of plasmids used to produce soluble human CD4 fragments can best be described with reference to Figure 2. As shown in Figure 2, plasmid vector pAc373/T4<sub>ex</sub>, which contains the truncated CD4 gene, was constructed from plasmids pAc373 and pSP65-T4. As described in detail in Example 1, a secreted form of the CD4 molecule was produced by releasing the CD4 cDNA insert contained in pSP65-T4. The CD4 cDNA insert was digested with NciI to produce a 1.17Kb fragment which lacks the ATG start codon and terminates just before the transmembrane region. The 1.17Kb fragment was ligated to a synthetic linker, with the result that either 371 residues (T4<sub>ex1</sub>) or

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370 residues ( $T4_{ex2}$ ) of the mature extracellular segment would be preserved.

Recombinant plasmids were produced and two (designated pAc373/ $T4_{ex1}$  and pAc373/ $T4_{ex2}$ ) were characterized in detail. The truncated CD4 cDNA constructs were integrated into the Autographa californica nuclear polyhedrosis virus (AcNPV) genome by homologous recombination, using known methods. Smith et al., Proc. Natl. Acad. Sci., U.S.A., 82:8404-8408 (1985). Baculovirus stocks were used to infect Spodoptera frugiperda (SF9) cells, which are publicly available. Subsequently, SF9 cells infected with the recombinant baculovirus containing the  $T4_{ex}$  cDNAs or wild type AcNPV were cultured in  $^{35}$ S-methionine and products were examined by SDS-PAGE, followed by autoradiography.

The  $T4_{ex1}$  polypeptide was shown to be the major secreted product of SF9 cells infected with the  $T4_{ex1}$  recombinant baculovirus. The predominant  $^{35}$ S labelled protein band in SDS-PAGE analysis of supernatants from SF9 cells obtained 54 hours after  $T4_{ex1}$  recombinant baculoviral infection was a 50KD band under reducing conditions. No CD4 material was precipitated from supernatants of wild type AcNPV-infected cells or detectable in the total supernatant.

Each of two representative  $T4_{ex2}$  preparations yielded a protein that migrated under reducing conditions with a molecular weight of 51KD. The different mobility (from that observed for  $T4_{ex1}$  protein) was not unexpected, given that  $T4_{ex2}$  contains 17 additional carboxy terminal amino acids derived from fusion with the polyhedrin gene.

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As described in Example 1, further analysis demonstrated that the 50KD T4<sub>ex1</sub> and 51 KD T4<sub>ex2</sub> proteins were the products of the CD4 gene. The soluble CD4 proteins produced in the baculovirus system were shown to bind to the HIV gp120 exterior glycoprotein, as described in Example 1, using two reciprocal co-precipitation experiments. Inhibition of HIV gp120 binding to CD4 + B4 lymphocytes by the T4<sub>ex1</sub> protein was also demonstrated, as were inhibition of HIV replication and inhibition of HIV envelope-induced syncytia, by the T4<sub>ex1</sub> and the T4<sub>ex2</sub> proteins (Example 1). As mentioned previously and as described in detail in Example 1, these effects were shown to be produced by the CD4 fragments without having a discernible effect on Class II MHC recognition events (e.g., they failed to inhibit CTL effector function), even in high concentrations. In addition, the soluble CD4 fragments were shown to have no discernible effect on Class II-directed physiologic T cell responses; they were shown to have no effect on proliferation of the T4+ tetanus toxoid specific Class II MHC restricted helper T cell clone CTT7 (Example 1).

Another approach was used to further analyze the specific physical interaction between T4<sub>ex</sub> proteins or their derived peptide fragments and HIV gp120. This approach is described in detail in Example 2. Briefly, this method made use of size fractionation of SDS-PAGE, followed by electroblotting of the T4<sub>ex2</sub> protein onto polyvinylidene difluoride membranes. Results of this work (see Example 2) showed that the single band of T4<sub>ex2</sub> protein at 50KD MW, when electrophoresed unreduced, was capable of binding HIV

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gp120 strongly. Conversely, identical quantities of T4<sub>ex2</sub>, reduced or reduced and amidomethylated, did not bind HIV gp120. Identical results were obtained with T4<sub>ex1</sub> proteins.

- 05 Enzymic fragmentation of the purified T4<sub>ex</sub> protein was also carried out. Results of papain digestion showed the presence of a fragment with a mobility of 28KD which binds HIV gp120. It was shown to bind HIV gp120 with the same efficiency as the
- 10 parent T4<sub>ex1</sub> protein and to be an intact polypeptide chain derived from the amino terminal region of the T4<sub>ex1</sub> protein. Similar experiments using trypsin fragmentation of T4<sub>ex2</sub> were also carried out to further define the nature of the HIV gp120 binding
- 15 fragments, as described in Example 2.

#### Modification of Biologically Active Soluble Human CD4 Fragments

- The cDNA sequence which encodes 370 amino acids of mature CD4 protein (T4 SEC1 cDNA) is represented
- 20 in Figure 1, as is the deduced amino acid sequence of the encoded CD4 protein. Modifications of the T4 cDNA as represented in Figure 1 have been made and the encoded soluble CD4 fragment expressed. Resulting CD4 fragments have been shown to bind to
- 25 HIV gp120 in vitro, as demonstrated by the ability to detect a complex between HIV gp120 and soluble CD4 proteins in solution.

- As explained previously and in Example 1, biologically active soluble human CD4 fragments
- 30 encoded by the nucleotide sequence of Figure 1 bind HIV gp120 and interfere with HIV infection of T cells without interfering with the function or



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proliferation of human T lymphocytes which are not infected with HIV.

Briefly, biologically active, modified soluble CD4 fragments are produced as described in the following paragraphs and as presented schematically in Figure 3. Detailed description of production of such fragments is presented in Example 3.

DNA encoding a soluble CD4 fragment is produced, either by using recombinant DNA techniques, such as excising it from a vector containing cDNA encoding such a fragment (see Example 1) or by synthesizing DNA encoding a soluble CD4 fragment mechanically and/or chemically, using known techniques.

In either case, the DNA obtained encodes a soluble CD4 fragment, capable of binding to the gp120 envelope protein of HIV in vitro, which includes none of the hydrophobic transmembrane region of CD4 or a portion of that region (generally six amino acids or less) small enough that it does not prevent solubilization of the fragment. In addition, the CD4 fragment is long enough (e.g., 10 amino acids or more) to bind effectively to HIV gp120 envelope protein.

Templates for subsequent mutagenesis are produced, using the CD4 fragment-encoding cDNA or DNA. As described below, this can be carried out using a single-stranded bacteriophage cloning vehicle, such as M13. This results in production of single-stranded DNA homologous to only one of the two strands of the DNA encoding the soluble CD4 fragment. The resulting single-stranded DNA is used as a template for producing the biologically active, modified soluble CD4 fragments, as follows:

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Oligonucleotides are produced, such that their sequence includes a base change (or changes) which, when incorporated into the nucleotide sequence of DNA subsequently used for the production of soluble CD4 fragments, results in a change in the encoded CD4 protein (i.e., different from that encoded by the nucleotide sequence of Figure 1). Such oligonucleotides are produced using standard methods. Oligonucleotides having a base change or base changes are referred to as mutagenized or mutant oligonucleotides.

The mutant oligonucleotide produced in this manner is hybridized to (e.g., by being kinased) the template produced as described above, to produce a template-mutant oligonucleotide complex, referred to as a mutant primer/template. The mutant primer/template is used for the production of a second strand of DNA, using well-known techniques. For example, synthesis of the second DNA strand is carried out by the Klenow fragment of DNA polymerase in the presence of dCTP $\alpha$ S. Taylor, J.W. *et al.*, Nucleic Acids Research, 13:8749-8764 (1985); Taylor, J.W. *et al.*, Nucleic Acids Research, 13:8764-8785 (1985); Nakayama, K. and F. Eckstein, Nucleic Acids Research, 14:9679-9698 (1986). The resulting strand of DNA contains a modification (or modifications) in the nucleotide sequence of T4 cDNA (i.e., is different from the nucleotide sequence represented in Figure 1) and is referred to as a mutant strand.

Unreplicated single-stranded DNA is removed and the double-stranded DNA is nicked with a selected restriction enzyme (e.g., NciI, which does not cut phosphorothioate DNA and, thus, does not cut the new

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DNA strand containing dCTP~~S~~ or the mutant strand). Nicked, nonmodified DNA is removed by digestion with another enzyme, such as exonuclease III. The resulting gapped DNA is repolymerized and, because  
05 the mutant strand serves as the template for repolymerization, the mutation or modification is copied into both strands.

Once produced, the double-stranded DNA, in which both strands contain the mutation or modification  
10 encoding the corresponding modification in the amino acid sequence of the soluble CD4 fragment is introduced into a competent host cell, such as a competent bacterial host (e.g., by transformation). The resulting plaques are grown and DNA contained in  
15 them is isolated, using known techniques, and sequenced to confirm the presence of the mutation.

The mutated DNA produced in this manner is excised from the M13 vector containing it, introduced into a suitable expression vector, such as CDM8, and  
20 transfected into an appropriate host cell, such as Cos cells, in which it is expressed. Aruffo, A. and B. Seed, Proceedings of the National Academy of Sciences, USA, 84:3365-3369 (1987). As a result, mutant CD4 proteins can be assayed, using known  
25 techniques. The vector-insert ligation mixture is introduced into competent host bacteria, such as the publicly available E. coli MC1061P3, and radiolabelled T4 DNA is used to identify CDM8 containing mutant T4 cDNAs.

30 Production, in Cos cells transfected with the vector containing mutant T4 cDNA, of modified soluble CD4 fragments capable of binding HIV (i.e., biologically active, modified soluble CD4 fragments)

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is subsequently assayed, using known techniques described below.

As a result of this procedure, double stranded DNA encoding a biologically active, modified soluble  
05 CD4 fragment is produced, the encoded CD4 fragment is expressed and its ability to bind the HIV gp120 envelope protein is assessed.

An alternative approach to producing a biologically active, modified soluble human CD4  
10 fragment of the present invention is to use peptide synthesis to make a peptide or polypeptide having the amino acid sequence of such a fragment.

This aspect of the subject invention will now be illustrated with reference to a specific  
15 modification, which is described in detail in Example 3 and production of which is represented in Figure 3. It is to be understood, however, that this is not meant to be limiting in any way and that other modifications can be made, using known techniques and  
20 the method of the present invention.

#### T4 cDNA and Templates for Mutagenesis

As represented in Figure 1, the T4<sub>ex</sub> cDNA was excised from the plasmid vector pAc373/T4<sub>ex</sub>, using the restriction enzyme BamHI. The ends of the  
25 fragments were blunted with DNA polymerase I and the fragment was ligated to XbaI linkers. The ligated fragment was cut with XbaI, excess linkers were removed and the linkered fragments were ligated to Xba-cut M13 (replicative form). M13 is a  
30 single-stranded bacteriophage cloning vehicle which has a closed circular DNA genome approximately 6.5Kb in size. Messing, J. and J. Viera, Gene, 19:269-276

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(1982). It is useful as a cloning vehicle in this context because infected cells release phage particles which contain single-stranded DNA which is homologous to only one of the two strands of cloned  
05 DNA and which can be used as a template. The resulting ligation mixture was transformed into competent TG1 host bacteria, which were plated out. The plaques were screened, using T4 oligonucleotides. Plaques hybridizing to sense oligonucleotides were  
10 selected and grown up to produce single-stranded M13 templates for mutagenesis.

#### Mutagenesis

Mutagenesis was carried out by the protocol which is marketed by Amersham and is based on the  
15 method of Eckstein (See Example 3).

Oligonucleotides whose sequence included a base change which, when incorporated, produced an amino acid change in the encoded CD4 protein (different from that encoded by the cDNA protein of Figure 1) were produced, using standard methods. In this case,  
20 a truncation of the CD4 molecule was introduced at amino acid #183. The normal T4 cDNA sequence is G-AAG-GCC-TCC-AGC-ATA-G (see Figure 1). An oligonucleotide having the sequence  
25 5'-G-AAG-GCC-TAA-AGC-ATA-G was synthesized. The difference in the two sequences is underlined. The serine encoded by the TCC of the normal T4 cDNA was modified to a stop codon (TAA) and the encoded modified protein terminated at this point (resulting  
30 in a cDNA fragment in which the terminal triplet is GCC and the terminal amino acid is alanine).

Production of Double-Stranded DNA

The modified oligonucleotide was kinased and hybridized to M13 T4 template, which served as a template for synthesis of a second strand of DNA, by  
05 the Klenow fragment of DNA polymerase in the presence of dCTP $\alpha$ S. Taylor, J.W. et al., Nucleic Acids Research, 13:8749-8764 (1985); Taylor, J.W. et al., Nucleic Acids Research, 13:8764-8785 (1985); Nakayama, K. and F. Eckstein, Nucleic Acids Research,  
10 14:9769-9698 (1986). This resulted in production of a strand of DNA (the second strand) containing a modification of the normal T4 cDNA nucleotide sequence (i.e., the sequence as represented in Figure 1). This modified strand is referred to as a mutant  
15 strand. Unreplicated single-stranded DNA was removed and the double-stranded DNA was nicked with the restriction enzyme, NciI. Because NciI does not cut phosphorothioate DNA, the new strand containing dCTP $\alpha$ S and the mutation was not nicked. The nicked,  
20 nonmodified DNA was removed by digestion with another enzyme (exonuclease III).

The gapped DNA was repolymerized using DNA polymerase I in the presence of T4 DNA ligase. Because the mutant strand served as the template, the  
25 mutation or modification was copied into both strands. The resulting double-stranded DNA was introduced into competent TG1 by transformation. Mandel, M. and A. Higa, Journal of Molecular Biology, 53:154 (1970). Derived plaques were grown up and  
30 single stranded and replicative form DNAs were isolated. The DNA was sequenced to confirm the presence of the mutation.

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05 Mutated DNA (DNA including the mutation introduced as a result of the DNA synthesis using the modified oligonucleotide, as described above) was excised from the replicative form of DNA with Xba and ligated to vector CDM8 which had been cut by Xba. The CDM8 vector is expressed in Cos cells upon transfection. Cos cells are a monkey kidney cell line, which have been transformed by simian virus 40 (SV40) DNA which includes the functional early gene region, and thus constitutively expresses the SV40 large T antigen, but has a defective origin of viral DNA replication. Gluzman, Y. et al., Cell, 23: 175-182 (1981). The CDM8 vector containing mutated DNA was transfected into Cos cells, in which it was expressed, thus making it possible to assay mutant CD4 proteins. The vector-insert ligation mixture was introduced into competent MC1061P3 host bacteria and CDM8 containing mutant T4 cDNAs, were identified by hybridization to radiolabelled T4 DNA. Ausubel, F.M. et al. (ed.), Current Protocols in Molecular Biology, Greene Publishing Associates, p. 1.4.9 (1988), Seed, B. and A. Aruffo, Proceedings of the National Academy of Sciences, USA, 84:3365-3369 (1987). Restriction enzyme analysis of mini-prep DNAs was used to determine the proper orientation of the insert in the CDM8 vector.

Determination of Ability of Modified Soluble CD4 Fragments to Bind HIV

30 Cos cells transfected with the CDM8 vector containing mutant T4 cDNA were assayed for production of modified soluble CD4 proteins capable of binding

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HIV, as described briefly in the following sections and in detail in the Exemplification.

Cos cells transfected with the mutant T4 cDNA-containing CDM8 vector were processed in order  
05 to produce dialyzed supernate, which was precleared with control rabbit anti-T cell receptor IgG coupled to a Sepharose support in order to minimize non-specific binding.

The precleared supernate was immunoprecipitated  
10 with a monoclonal anti-CD4 antibody (19Thy5D7) coupled to a Sepharose support. 19Thy5D7 is an antibody against a T4 epitopic site which competes with HIV for binding of gp120. Thus, binding of a component of the supernate to 19Thy5D7 is suggestive  
15 of the presence in the supernate of a component capable of binding HIV.

The ability of the modified soluble CD4 fragments produced in this manner to bind to the HIV exterior gp120 glycoprotein can be directly  
20 determined as follows:

Labelled and dialyzed Cos supernates determined to contain optimal levels of recombinant, secreted CD4 protein will be taken for co-precipitation studies. For example, 67 ng of gp120 (1 ul at 67  
25 ug/ml in PBS/0.1% BSA) can be added to 0.5 ml of Cos supernates. As a control, no addition is made to a second 0.5 ml aliquot of supernate. After a 2 hour incubation at 37°C, 500 ng of monoclonal anti-gp120 are added to both supernates, followed by rabbit  
30 anti-mouse IgG coupled to Sepharose 4B (10 ul). The samples are then rotated for 2 hours at 4°C. The beads are then wash twice with 100 ul cold PBS and eluted with non-reducing SDS sample buffer (30 ul).



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Aliquots are run on 12.5% non-reduced SDS-PAGE, followed by autoradiography. T4<sub>ex1</sub> protein (protein encoded by the modified T4 DNA) produced in the Cos system can be readily co-precipitated with anti-gp120 antibody in the presence of gp120. Rabbit heteroantisera to the CD4 protein (T4<sub>ex1</sub>) is also available for identification of modified CD4 products in which monoclonal CD4 epitopes are no longer present. Thus, this makes it possible to be certain that CD4-related protein is being translated in Cos even in the absence of gp120 binding material. Co-precipitation of gp120 with T4<sub>ex1</sub> protein produced in Cos is readily detected in the presence of anti-gp120 antibody plus rabbit anti-mouse Ig. The co-precipitated product will be a 50KD band in SDS-PAGE analysis after autoradiography. The fact that no equivalent <sup>35</sup>S-cysteine labelled T4<sub>ex1</sub> band is detected in the absence of gp120 demonstrates the specificity of this reaction.

The modified soluble CD4 protein produced as described above includes the amino acid sequence (as shown in Figure 1) of the CD4 protein through amino acid 183. In addition to its ability to bind to HIV and, thus, interfere with infection of cells by the virus, this truncated soluble CD4 protein has the further advantage that it lacks the glycosylation sites present in T4<sub>ex1</sub> and should, thus, be less immunogenic. In addition, the terminal amino acid (histidine) present in the mature CD4 protein encoded by the nucleotide sequence of Figure 1 is absent from the biologically active, modified soluble CD4 fragment of the present invention. It is also absent in the native CD4 molecule. Because the

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glycosylation sites are not present in the CD4 fragment produced in this manner, fragments of this type can be expressed in a bacterial host.

Immunoprecipitation of CD4 protein having amino acid  
05 residues 1-182 (as represented in Figure 1)

identifies a band of approximately 19 kD on SDS-PAGE of 19Thy5D7 immunoprecipitates from transfected Cos supernatants. Co-precipitation studies with gp120 and anti-gp120 antibody identifies the same band.

10 As explained previously, there are many possible useful modifications (e.g., changes in amino acid sequence, truncation) of CD4 protein which can be produced as described above for truncation of the protein at amino acid 183. Some of these

15 modifications have been described above. Additional modifications can be made at other sites within the CD4-encoding DNA, with the outcome that expression of the modified DNA will result in production of modified soluble human CD4 fragments. Biological  
20 activity (e.g., ability to bind HIV gp120 and interfere with HIV infection of cells) can be assessed as described herein.

For example, truncation at a different amino acid can be carried out. In one case, truncation of  
25 T4<sub>ex1</sub> after amino acid 369 (i.e., removal of the carboxy terminal histidine) is carried out in a similar manner, by insertion of a termination codon (see Figure 1). It is reasonable to expect that the resulting truncated form will retain the capability  
30 of binding HIV. In addition, such a modified form has the advantage that it lacks the histidine present in the CD4 protein encoded by the cDNA of Figure 1 and not present in the native molecule.

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Another approach is to produce biologically active, modified soluble CD4 fragments in such a manner that fragments which include of one or more domains of the encoded protein are obtained.

05        Production of fragments of the present invention in which one or more of the domains is present is of interest, for example, because of the importance of at least the first two domains (see Figure 7) in binding of CD4 with HIV. That is, it is known that  
10 the external segment of CD4 (T4) functions as the T cell surface receptor for HIV, by binding the major HIV coat protein (gp120) with relatively high affinity.

However, the region of the CD4 molecule that  
15 binds gp120 has not yet been defined. Nor is it known whether the same or different segments of CD4 bind to an invariant region of class II MHC molecules(s) which are the presumed physiologic CD4 ligand. Meuer, S. et al., Proceedings of the  
20 National Academy of Sciences, USA, 79:4395 (1982); Biddison, W. et al., Journal of Experimental Medicine, 156:1065 (1982); Gay, D. et al., Nature, 328:626 (1987). In this regard, sequence analysis of CD4 has suggested an evolutionary origin from a  
25 structure with four immunoglobulin-related domains (Figure 7A). Two of these domains (the first two) are involved in HIV gp120 binding. The NH<sub>2</sub>-terminal CD4 domain (amino acids 1-92), termed domain 1, bears the most structural homology to Ig light chain  
30 variable regions (about 32% at amino acid level). Eight of 14 invariant residues are conserved between domain 1 of T4 and V<sub>Kappa</sub> domains. Maddon, P. et al., Cell, 42:93 (1985). The first and second

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cysteines (amino acids 16 and 84; Figure 7A and 7B) in domain 1 of the CD4 sequence are separated by 67 amino acids, which are positions and spacings similar to members of the Ig family. By analogy to sheep and mouse CD4, these cysteines in human CD4 also form a conserved intrachain disulfide bond characteristic of V domains. In addition, secondary structural analysis suggests the presence of seven beta strands within the CD4 domain 1. Cysteines bridged by intrachain disulfides form the boundaries of hypothetical domains 2 (amino acids 120 and 151) and 4 (amino acids 303 and 345), with certain short stretches of Ig-like sequences clustered around them. In contrast, no cysteines are found in domain 3, although the latter bears homology by sequence alignment with a poly Ig receptor.

The NH<sub>2</sub>-terminal region of CD4, including the immunoglobulin V-like domain, has been shown to be required for gp120 interaction. In contrast, the carboxy terminal half of the molecule containing the two potential N-glycosylation sites does not appear to be necessary.

For example, insertion of a TAA termination codon after the valine codon at position 128 (GTG, Figure 1) will result in production of a domain 1 T<sub>4</sub><sub>ex1</sub> construct. A domain 1 T<sub>4</sub><sub>ex1</sub> construct and a partial domain 2 mutant will be obtained if a TAG termination codon is inserted after the fourth cysteine, which produces truncation after amino acid 162 (Figure 1). This will produce a modified soluble CD4 fragment capable of binding HIV and will also make manufacture/production easier because of

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improved ability to introduce the construct into a cell line.

In a similar manner, production of a domain 1, 2 and partial domain 3 construct can be carried out.

05 In this case, the triplet encoding the glutamine at amino acid position 243 (see Figure 1) will be altered to a TAG termination codon. This will result in production of a modified soluble CD4 protein having the same advantages described above for the  
10 protein resulting from termination after the fourth cysteine.

The method described herein can be used, with appropriate modification, to convert asparagine and N-linked glycosylation sites at positions 271 and 300  
15 to aspartate. This can be carried out in the same construct or in two separate constructs (each including one of the two modifications). In either case, the two codons at the positions indicated will be modified: in the case of the codon for amino acid  
20 271, to GAC and in the case of the codon for amino acid 300, to GAT. This modified protein will also bind HIV and has the further advantage that because the glycosylation sites are no longer present, it will be less immunogenic than a fragment which  
25 includes such sites.

Additional modification of the mature CD4 protein can similarly be made, as desired, and subsequently shown to have the capability of binding HIV by the means described herein. Expression of  
30 additional constructs (DNA encoding additional modified soluble CD4 fragments) will be carried out, for example, in baculovirus (e.g., Autographa

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californica), Chinese hamster ovary (CHO) cells or E. coli.

In the case of production in baculovirus, this will be carried out as follows and in a similar manner to that described by Hussey et al. and Smith et al., the teachings of which are incorporated herein by reference. Hussey, R.E. et al., Nature, 331: 78-81 (1988); Smith G., et al., Proceedings of the National Academy of Sciences, USA, 82: 8404-8408 (1985).

Transfer of the T4<sub>ex1</sub> sequence from the plasmid vector to the Autographa californica nuclear polyhedrosis virus (AcNPV) genome can be accomplished essentially as described in Smith et al., (1985) Proceedings of the National Academy of Sciences, USA, 82:8404-8408. Cotransfection by calcium phosphate precipitation of 4 ug pAc373/T4<sub>ex1</sub> DNA with 1 ug of purified AcNPV DNA into Spodoptera frugiperda (SF9) cells, which are publicly available, results in homologous recombination between the recombinant sequence of the transfer vector and the polyhedrin gene sequence of AcNPV. Recombinant AcNPV contains an inactivated polyhedrin gene which no longer forms occlusions in infected cells, thus providing a means by which infected and noninfected cells can be distinguished. For plaque purification, 2 x 10<sup>6</sup> SF9 cells can be seeded in 100 mm petri dishes 24 hours prior to assay. Ten fold dilutions of viral supernatant are prepared, using final media (Grace's insect medium (Gibco, Grand Island, NY), TC yeastolate 0.33%, lactalbumin hydrosylate 0.33%, 2 mM supplemental glutamine and 50 ug/ml gentamycin containing 10% FCS (Hyclone, Logan, UT). Each plate

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is inoculated with virus (e.g., 1 ml.,  $10^{-3}$  to  $10^{-7}$  dilution) plus 2 ml of final media. After incubation for 2 hours, the inoculum is removed and replaced with 10 ml of 1.5% Sea Plaque agarose (FMC

- 05 Bioproducts, Rockland, ME) in final media. Plates are transferred to a humid environment after agarose solidification for 4-6 days at  $27^{\circ}\text{C}$ .

- Plaque assay of the transfection supernatant will demonstrate distinct morphological differences  
10 between infected cells; infected cells which are occlusion positive contain wild type AcNPV and infected cells which are occlusion negative contain recombinant CD4 virus. Occlusion-negative plaques are identified, selected, and further plaque  
15 purified. DNA from cells infected with putative CD4 recombinant virus will be hybridized with a  $^{32}\text{P}$  labelled CD4 cDNA probe to verify the presence of the CD4 sequence.

- Production of the  $\text{T4}_{\text{ex}}$  polypeptide is carried  
20 out as follows: SF9 cells ( $6 \times 10^5$  cells per well) are seeded per well in 24 well Nunc plates (Interlab, Thousand Oaks, CA) for 2 hours at  $27^{\circ}\text{C}$  and then adherent SF9 cells are infected with virus at an MOI of 10 in 0.2 ml final media for 2 hours. The  
25 inoculum is then removed and cells are cultured in 0.5 ml fresh medium at  $27^{\circ}\text{C}$  for 48 hours. Adherent cells are then washed twice with 0.5 ml Grace's medium lacking serum and methionine. This is followed by incubation in 0.5 ml in the same medium  
30 for 1 hour. The adherent cells are washed once and then cultured for 6 hours in serum and methionine-free Grace's medium containing  $67 \text{ uCi } ^{35}\text{S}$  methionine (New England Nuclear, Boston, MA 1134

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Ci/nmol). Culture supernatants are harvested, microfuged for 10 minutes, and dialyzed at 4°C against PBS containing 0.5% sodium azide and 10 mM cold methionine. Cells are dislodged from the wells, 05 washed twice with Grace's medium at 4°C (by centrifugation in a Sorvall RT6000 for 5 min at 1000 rpm) and finally lysed for 30 min at 4°C by the addition of a RIPA buffer containing 1% Triton X-100, 0.15 M NaCl and a cocktail of protease inhibitors, as 10 described below. The lysates are microfuged for 10 min and dialyzed at 4°C using the same procedure as was used for culture supernatants.

Both lysates and culture supernatants are subjected to immunoprecipitation for 16 h at 4°C with 15 a monoclonal anti-CD4 antibody (19Thy5D7) linked to Affigel-10 beads (5 mg monoclonal antibody/ml gel). After immunoabsorption, the beads are washed twice with lysis buffer and bound material is eluted by treatment of the beads with 0.1M glycine-HCl buffer, 20 pH 2.0. Eluates and whole samples of lysates or culture supernatants are mixed with SDS sample buffer containing 2-mercaptoethanol, boiled for 5 minutes and electrophoresed in 12.5% mini-slab gels according to Laemmli. Laemmli, Nature, 227:680-685 (1970). 25 Subsequently, the gels are fixed, dried and autoradiographed using Kodak XAR-5 film.

High titer viral stocks are generated by infecting SF9 cells at an MOI of 1 and culturing at 1 x 10<sup>6</sup> cells/ml for 4 days in final media. These 30 stocks are used for infecting SF9 cells for production of protein. For large scale production of protein, SF9 cells are grown in 2 liter spinner flasks in final media. Cells are harvested and



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infected with an MOI of 15 (using high titer viral stocks) at a concentration of  $10 \times 10^6$  cells/ml.

Cells are then pelleted, resuspended in media at  $1 \times 10^6$ /ml, and cultured for 3 days at 27°C in  
05 spinner flasks. At this time, supernatants are collected by centrifuging cultures to remove cells.

For large scale purification, infected SF9 cell culture supernatants are harvested by centrifugation of cells in a Sorvall H-4000 rotor at 800 rpm for 6  
10 minutes at 4°C. The culture supernatants are then subjected to protease inhibition by the addition of a cocktail of protease inhibitors made up of leupeptin, antipain, pepstatin, and chymostatin to final concentrations of 0.5 ug/ml; soybean trypsin  
15 inhibitor to 0.02 ug/ml; and phenyl methyl sulfonyl fluoride (PMSF) to 1.25 mM, followed by adjustment of the pH to 6.8 by the dropwise addition of 1 M NaOH. The samples are subsequently clarified by centrifugation in a Sorvall GSA rotor at 8000 rpm for  
20 25 minutes at 4°C and pumped at 4°C at a flow-rate of 30 ml/hour through a 2 ml precleared immunoabsorbent column, 21Thy2D3 monoclonal antibody (anti-T8) coupled to Affigel-10 (Biorad), followed in series by a 7 ml column of anti-CD4 monoclonal antibody  
25 (19Thy5D7) coupled to Affigel 10 at a concentration of 7.5 mg monoclonal antibody per ml of gel. The monoclonal antibodies are made according to conventional methods. The anti-CD4 column is then washed with 30 ml of 10 mM Tris-HCl buffer, pH 6.3  
30 followed by 15 ml of 0.1M glycine-HCl, pH 5.0. The bound CD4 polypeptides are eluted by pumping 0.1 M glycine-HCl, pH 2.0 through the washed anti-CD4 column and 0.8 ml fractions of eluant are collected

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into tubes containing 0.15 ml 1 M Tris-HCl, pH 7.5. During the whole column fractionation procedure, eluate absorption is monitored at 280 nm with a Uvicord 2 (LKB, Gaithersburg, MD) fitted with an event marker. Fractions of neutralized pH 2.0 eluate containing protein are pooled and concentrated by ultrafiltration in a stirred cell (Amicon, model 3) fitted with a YM-5 membrane. Typically the yield of purified T4<sub>ex</sub> polypeptides is approximately 1 ug/ml of infected SF9 culture supernatants. Aliquots containing 1 ug of protein concentrate (assuming that 1 OD unit = 1 mg/ml at a 280 nm) are examined for purity in 12.5% SDS-polyacrylamide slab gels, followed by staining with Coomassie blue.

Polypeptides produced in this manner, can be purified and characterized using known methods, to confirm that they are, in fact, those encoded by the modified CD4 cDNA introduced into the cells as described.

Alteration of Ability of CD4 Fragments to Bind HIV gp120

Regions or sites on human CD4 critical for HIV gp120 binding were identified, as described below and, based on the identification of critical sites, soluble human CD4 fragments with altered HIV gp120 binding ability were produced, as is described below, particularly in Examples 4 and 5.

Identification of Amino Acid Residues of Human CD4 Critical for gp120 binding

The extracellular segment of murine CD4 is overall 50% identical to its human counterpart

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(Maddon, P.J., et al., Proc. Natl. Acad. Sci. USA,  
84: 9155-9159 (1987) at the amino acid (a.a.) level  
but fails to bind gp120. McClure, M.O., et al.,  
Nature, 330: 487-489 (1987) These differences were  
05 used in precisely defining those residues of human  
CD4 critical for gp120 binding. Substitutions of  
all non-conserved murine for human CD4 residues  
between amino acid positions 27-167 were made. To  
this end, oligonucleotide-directed mutagenesis was  
10 used to create each of 16 individual mutant human CD4  
molecules containing from 1 to 4 amino acid  
substitutions. Introduction of as few as three amino  
acids into corresponding positions of human CD4  
resulted in CD4 fragments unable to bind gp120.  
15 These critical residues have been shown to be located  
in domain I as well as in domain II of CD4, thus  
implying that the gp120 binding site is complex and  
involves both of the NH<sub>2</sub>-terminal domains. Modelling  
studies using the 3-dimensional coordinates of the V<sub>H</sub>  
20 Bence-Jones homodimer, REI, localize the site of  
domain I to the C'  $\beta$  strand. Thus, domain I is  
distant from the loops analogous to hypervariable  
regions.

Residues of the CD4 structure involved in HIV  
25 gp120 binding were characterized through use of a  
Cos-1 cell expression system and a cDNA encoding the  
anchor-minus CD4 segment termed T4<sub>ex1</sub>. Hussey, R.E.,  
et al., Nature, 331: 78-81 (1988) The 370 amino acid  
T4<sub>ex1</sub> protein (Figure 1) contains 369 of the  
30 predicted 372 NH<sub>2</sub>-terminal amino acids of the CD4  
extracellular segment and a COOH-terminal histidine.  
As shown in Figure 8, this structure is comprised of  
three intrachain disulfide bonded domains (a domain

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is defined as residues between and including 20 amino acid residues to either side of the cysteines), and one domain (III) which lacks cysteine residues but, like its counterparts, is immunoglobulin-like.

- 05 Clark, S., et al., Proc. Natl. Acad. Sci. USA, 84:  
1649-1653 (1987). Nanomolar concentrations of T<sub>4</sub><sub>ex1</sub>  
inhibit gp120-transmembrane CD4 interaction,  
syncytium formation and HIV infection by binding to  
gp120-expressing cells. Hussey, R.E., et al.,  
10 Nature, 331: 78-81 (1988).

- As described in Example 4, the T<sub>4</sub><sub>ex1</sub> construct  
was subcloned into the vector CDM8 and transfected  
into Cos-1 cells. Seed, B., et al., Proc. Natl.  
Acad. Sci. USA, 84: 3365-3369 (1987) Supernatants  
15 from metabolically labelled transfected cells were  
tested by immunoprecipitation with an anti-CD4  
monoclonal antibody (19Thy5D7). The resulting  
precipitate was subjected to SDS-PAGE. Results  
showed the presence of a 50KD CD4-derived molecule in  
20 transfected Cos-1 cell supernatants (Figure 9, lane  
3). The same molecule is co-precipitated from Cos-1  
supernatants with an anti-gp120 monoclonal antibody  
after preincubation of the supernatant with gp120  
(Figure 9, lane 5). These reactions are specific for  
25 T<sub>4</sub><sub>ex1</sub>, as demonstrated by the fact that 1) an  
irrelevant antibody (anti-T5) fails to precipitate  
T<sub>4</sub><sub>ex1</sub> (Figure 9, lane 1) and 2) no CD4 band is  
detected with anti-gp120 antibody in the absence of  
gp120 (Figure 9, lane 6).

- 30 Prior studies, described above, employing either  
CD4 DNA truncation or proteolytic digestion  
demonstrated that the residues critical for gp120  
interaction reside in domains I and/or II

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- exclusively. Trauneker, A., et al., Nature, 331: 84-86 (1988); Berger, E.A., et al., Proc. Natl. Acad. Sci. USA, 85: 2357-2361 (1988) (Richardson, N.E., et al., Proc. Natl. Acad. Sci. USA, in press)
- 05 Similarly, the Cos-1 cell derived product of a T<sub>4</sub><sub>ex1</sub> protein truncated after amino acid residue 182 (by insertion of a stop codon in the cDNA sequence) is precipitated as a 20KD protein by anti-CD4 antibody and binds to gp120 (Figure 9, lanes 4 and 7,
- 10 respectively). In contrast, expression of a cDNA truncated at amino acid 110 (containing domain I only) failed to give rise to a gp120 binding protein. (Example 4) These data suggest that both domains I and II are required for HIV gp120 binding.
- 15 Therefore, further analysis of the CD4-gp120 interaction was carried out by creating 35 amino acid substitutions which encompass all non-conservative mouse-human species differences within the first two domains of CD4 between amino acid residues 25 and
- 20 167. The NH<sub>2</sub>-terminal CD4 amino acids were not considered here because an NH<sub>2</sub>-terminal synthetic peptide failed to block HIV gp120 binding, even at millimolar concentrations. For each substitution, an amino acid of the human sequence was replaced with
- 25 the amino acid found in the equivalent position of the murine CD4 sequence. Maddon, P.J., et al., Proc. Natl. Acad. Sci. USA, 84: 9155-9159 (1987). The murine CD4 sequence does not bind gp120, and, thus, it was anticipated that some murine substitutions
- 30 would abrogate human CD4-gp120 interaction. As shown in Table 1, 15 oligonucleotides were used in a standard site-directed mutagenesis protocol, as described in Example 4, to produce 16 different

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versions of the human CD4 molecule containing from  
104 substitutions each. The positions of these  
substitutions are listed in Table 1 and  
diagrammatically mapped in Figure 8. All 16 CD4  
05 mutants were assayed after transfection into Cos-1  
cells by immunoprecipitation with anti-CD4 monoclonal  
antibody and by gp120 co-precipitation with  
anti-gp120.

TABLE 1. PRODUCTION AND ANALYSIS OF CD4 SITE-DIRECTED MUTANTS

Mutant	Oligonucleotide used for mutagenesis	Amino acid change	Anti-CD4 immuno-precipitation	Anti-gp120 co-precipitation
M1A	Mouse subst. T 223 CAA-TTC-ACC-TGG-AAA-TTC-TCC-GAC-CAG-AGA-AAG 255 Human aa H N H I	aa 27 H to T aa 30 H to F aa 32 H to D	+	+
M1B	T F D R 223 CAA-TTC-ACC-TGG-AAA-TTC-TCC-GAC-CAG-AGA-AAG 255 H N H I	aa 27 H to T aa 30 H to F aa 32 H to D aa 34 I to R	+	+
M2	H 261 G-GGA-AAT-CAC-GCC-TCC 276 Q	aa 40 Q to H	+	+
M3	G P S 283 ACT-AAA-CGT-GGA-TCC-CCG-AGT-AAT-GAT-GG 311 F K L	aa 48 P to G aa 50 K to F aa 51 L to S	+	-
M4	K 333 GG-CAC-AAA-GGA-AAC-TTC 351 Q	aa 64 Q to K	+	+
M5	H K 355 CTG-ATC-ATC-AAT-AAG-CTT-AAG 375 K H	aa 72 K to H aa 73 H to K	+	+
M6	Q 382 GAC-TCA-CAG-ACT-TAC-ATC 399 D	aa 80 D to Q	+	+
M7	H R E 408 CTG-GAC-AAC-CCG-AAG-GAG-GAG-CTG-GAA-TTC-C 436 D Q Q	aa 88 D to H aa 89 Q to R aa 94 Q to E	+	+
M8	K P S 436 CTA-GTG-TTC-AAA-TTC-ACT-GCC-AAC-CCT-GAC-ACC-ACC-CTG-CTT-C 478 C S H	aa 99 G to K aa 104 S to P aa 107 H to S	+	+
M9	S K V 499 ACC-TTG-GAG-AGC-AGC-AAG-GTT-AGT-AGC-CCC 528 P P G	aa 121 P to S aa 122 P to K aa 123 G to V	+	- <sup>c</sup>
M10	L T E 520 ACT-ACC-CCC-CTA-ACC-CAA-TGT-ACG 543 S V Q	aa 127 S to L aa 128 V to T aa 129 Q to E	+	+
M11	H K V 534 G-CAA-TGT-AGC-CAT-AAA-AGG-GGT-AAA-CTC-ATA-CAG-GC 569 S P H	aa 132 S to H aa 133 P to K aa 137 H to V	+	+
M12	V 570 G-CGG-AAG-GTC-CTC-TCC-G 586 T	aa 143 T to V	+	+
M13	R 590 CT-CAG-CTG-CCG-CTC-CAG-G 607 E	aa 150 E to R	+	+
M14	D P H 606 G-GAT-AGT-GAC-TTC-TGG-AAT-TGC-ACT-GTC 633 G T T	aa 155 G to D aa 156 T to P aa 158 T to H	- <sup>b</sup>	- <sup>c</sup>
M15	T L D 626 CC-ACT-GTC-ACC-CTG-GAC-CAG-AAC 648 L Q H	aa 162 L to T aa 163 Q to L aa 164 H to D	+	+

<sup>a</sup>Two mutants were recovered from the mutagenesis using this oligonucleotide; one contained mutations at amino acid 27, 30 and 32 but not 34 and the second contained all four changes. These two mutants were transfected separately.

<sup>b</sup>M14 was also negative when tested for immunoprecipitation with anti-CD4 monoclonal OKT4A

<sup>c</sup>A very faint 50KD band (~10 fold less intense than T<sub>4</sub><sub>ex1</sub>) was observed upon coprecipitation with gp120.

Mutagenesis, immunoprecipitation and coprecipitation procedures are described in the legend to Fig. 1.

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Immunoprecipitation of the original T4<sub>ex1</sub> and four representative mutants is shown in Figure 10 (panel a). In addition to T4<sub>ex1</sub>, each of the mutants M5, M10, M7 and M3 react with the anti-CD4 monoclonal antibody 19Thy5D7. As shown in Table 1, 15 of the 16 mutants react with anti-CD4 antibody. Only mutant M14 did not react; it was also unreactive with OKT4A, which is a second monoclonal antibody directed at a different CD4 epitope.

Thirteen of the 16 mutants bound gp120 in a manner equivalent to T4<sub>ex1</sub>, as judged by the co-precipitation assay. Figure 10 (panel b) demonstrates that T4<sub>ex1</sub>, M5, M10 and M7 are all co-precipitated by anti-gp120 in the presence of gp120. Overall, a 2-3 fold experimental variation in co-precipitation with gp120 was observed (T4<sub>ex1</sub> vs. M5 in panel b Figure 10). Among gp120-binding CD4 proteins, however, a positive signal was observed in every experiment (using a minimum of 2-3 separate transfections). In contrast, although M3 is recognized by anti-CD4 antibody, it fails to bind to gp120 (Figure 10, panel b). In addition, M9 (Table 1) has a substantially reduced gp120 binding capacity, although anti-CD4 monoclonal antibody immunoprecipitates a band of identical size and intensity to T4<sub>ex1</sub>. M3 contains three amino acid substitutions in human CD4 domain I at positions 48, 50 and 51. One or more of these changes clearly abrogates the ability of CD4 to bind to HIV gp120. M9 contains three amino acid substitutions in domain II of CD4 at positions 121-123. Thus, alteration of a few residues in either CD4 domain I or domain II results in abrogation of HIV gp120 binding.



In addition, M14 demonstrates reduced binding to gp120 (Table 1). M14 also failed to bind to the two anti-CD4 monoclonal antibodies examined. Thus, one cannot rule out the possibility that the three  
05 substitutions in M14 (at positions 155, 156 and 158) somehow decrease the expression of this mutant CD4 protein. It is more likely that these substitutions have destroyed both the gp120 binding site and the epitopes recognized by the two monoclonal antibodies,  
10 perhaps through a general disruption of the CD4 protein's 3-dimensional structure because translation of in vitro transcribed RNA from M14 gave results identical to T4<sub>ex1</sub> transcribed RNA.

The contribution of CD4 domain I to gp120  
15 binding was recognized previously in studies of the T4<sub>ex1</sub> polypeptide produced in a baculovirus system in conjunction with proteolytic fragmentation analysis, microsequencing and a specific CD4-gp120 binding assay. Richardson, N.E., et al., Proc. Natl. Acad.  
20 Sci. USA. Richardson and co-workers showed that disruption of the peptide bond at lysine 72 by tryptic cleavage destroyed CD4-gp120 interaction without inducing any detectable alterations in other domains of CD4. Furthermore, reduction of intrachain  
25 disulfide bonds in the CD4 molecule also abrogated high affinity gp120 binding, thereby strongly implying that the binding site for gp120 is complex and depends on the stabilized CD4 structure. Whether the domain I and II mutations introduced in the work  
30 described herein affect gp120 contact residues themselves or, alternatively, affect the tertiary structure around the contact residues cannot be concluded at present. Footprint analysis of

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CD4-gp120 protein-protein interactions or analysis of CD4-gp120 cocrystals will be necessary to determine the effect of the mutations described. Nevertheless, the ability of a synthetic peptide comprising amino acid residues 23-56 to inhibit syncytium formation at  $10^{-4}$  M may support the notion that residues 48, 50 and/or 51 contribute to the gp120 binding sites. Jameson, B.A., et al., Science, 240: 1335-1339 (1988)

Eight residues are conserved between domain I of CD4 and the 14 invariant residues of the Kappa light chain variable (V) regions. Maddon, P., et al., Cell, 42: 93-104 (1985). In addition, the first and second cysteines (amino acids 16 and 84) in domain I of CD4 are separated by 67 amino acids, positions and spacing similar to those of members of the immunoglobulin family. Furthermore, secondary structural prediction suggests the presence of eight Kappa strands in CD4 domain I. In light of these homologues to Ig, CD4 domain I was modelled on the basis of the known 3-dimensional coordinates of the  $V_k$  Bence-Jones homodimer, REI. Use of this model has resulted in accurate prediction of each of three tryptic cleavage sites in domain I to be surface exposed, thus supporting the validity of the CD4 model. Richardson, N.E., et al., Proc. Natl. Acad. Sci. USA. It was therefore of interest to determine the relative positions of the M3 mutations at amino acid residues 48, 50 and 51 of CD4.

The region of residues in the alpha carbon skeleton of the REI homodimer corresponding to the mutated CD4 residues which abrogate gp120 binding were determined. This region corresponds to the C' strand unique to V domains which connect the two

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sheets. Williams, A.F., et al., Ann. Rev. Immunol.,  
6, 381-405 (1988). The alignment between REI and CD4  
requires a gap in this segment, and, thus, it is not  
meant to imply that the CD4 alpha carbon skeleton  
05 follows an identical course in this region.  
Nevertheless, it is very likely that the CD4 sequence  
will loop out and be solvent exposed. Furthermore,  
it should be noted that this site is distinct from  
the three segments equivalent to the hyper-variable  
10 loops of the REI homodimer.

Based on the above analysis, one prediction  
would be that if gp120 does contact residues in the  
region analogous to the C' strand of REI, it might  
also contact residues in CD4 domain II adjacent to  
15 this region. Perhaps M9 and/or M14 mutations are  
localized to such sites. That domains I and II of  
CD4 might be spatially close to one another in some  
regions is further supported by antibody competition  
studies in which an antibody (OKT4A) whose epitope  
20 was mapped to a region in domain I showed reciprocal  
competitive binding with two antibodies (OKT4F and  
OKT4B) whose epitopes mapped to domain II. Jameson,  
B.A., et al., Science, 240: 1335-1339 (1988).

The region of CD4 domain I implicated as a  
25 possible binding site for gp120 is distinct from the  
loops analogous to hypervariable complementarity  
determining segments. If those loops form a binding  
site for class II MHC, the putative natural ligand of  
CD4 one can speculate that gp120 may be incapable of  
30 inhibiting class II recognition events, even after  
binding to the CD4 structure. Krensky, A.M., et al.,  
Proc. Natl. Acad. Sci. USA, 79: 2365-2369 (1982);  
Meuer, S.C., et al., Proc. Natl. Acad. Sci. USA, 79:

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4395-4399 (1982); Biddison, W., et al., J. Exp. Med.,  
156: 1064-1076 (1982); Marrach, P., et al., J. Exp.  
Med., 158: 1077-1091 (1983); Doyle C., et al.,  
Nature, 330: 256-259 (1987) The CD4 mutants

05 described herein should be useful in future analysis  
of CD 4- class II MHC interactions.

As a result of the identification of sites  
critical to binding of CD4 to the HIV gp120 envelope  
protein, it is now possible to produce modified  
10 soluble human CD4 fragments whose ability to bind  
gp120 is altered (i.e., whose ability to bind gp120  
is different from that of the corresponding  
naturally-occurring human CD4 fragment). As  
described in the previous sections and in Examples 4  
15 and 5, such sites have been identified by  
oligonucleotide-directed mutagenesis used to create  
16 mutant human CD4 molecules which resulted in  
substitution of all non-conserved murine amino acid  
residues for human CD4 residues between amino acid  
20 positions 27-167, as represented in Figure 1.

As shown in Table 1, 15 of the 16 CD4 "mutants"  
created as described react with anti-CD4 monoclonal  
antibody 19thy5D7 and 13 of the 16 bind gp120 in a  
manner equivalent to the gp120 binding evidenced by  
25 T<sub>4</sub><sup>ex1</sup>. Three mutants, designated M3, M9 and M14, do  
not exhibit gp120 binding equivalent to that of  
T<sub>4</sub><sup>ex1</sup>: M3 fails to bind gp120; M9 has substantially  
reduced gp120 binding capacity; and M14 demonstrates  
reduced gp120 binding ability. As also shown in  
30 Table 1, M3 and M9 are recognized by anti-CD4  
antibody and M14 is not recognized by either of the  
two anti-CD4 antibodies used.

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These results demonstrate that these sites are critical for gp120 binding by CD4 and that the changes made in the amino acid sequence of human CD4 (as represented in Figure 1) to produce these CD4 mutants resulted in elimination of or reduction in gp120 binding. In a similar manner, other changes at one or more of these critical sites can result in elimination of or reduction in gp120 binding ability. Conversely, amino acid residues can be introduced at these critical sites to produce modified soluble human CD4 fragments with enhanced gp120 binding ability.

Such substitutions can be made: 1) at one, two or all three of the critical sites (i.e., at one or more of the three amino acid sites represented by mutants M3, M9 and M14) and/or 2) of one, two or all three amino acid residues within each site (i.e., within a critical site, of amino acid residues 1, 2 or 3 individually, 1, 2 and 3 in any combination of a 2 amino acid residues; or of all three amino acid residues).

For example, in mutant M3, glycine, proline and serine, respectively, replace proline, lysine and leucine, which occur at amino acid positions 48, 50 and 51 of human CD4. Substitution of one or more of those amino acids by other amino acids of the same type (e.g., at position 48 by another amino acid with a nonpolar R group) as that present at that position in M3 can be made and the effect on gp120 binding ability determined.

Substitutions at these three sites, individually or in combination, of amino acids having characteristics different from those of amino acid

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whose presence at those sites has been shown to eliminate or reduce gp120 binding ability can also be made and their effect on binding ability assessed using the anti-CD4 immunoprecipitation and anti-gp120  
05 coprecipitation methods described in the Examples. In particular, substitutions of some or all of the amino acids at one or more of these critical sites which result in modified soluble CD4 fragments with enhanced gp120 binding ability can be made. Using  
10 the techniques described herein, CD4 fragments having enhanced binding ability can be identified.

One approach to producing modified soluble human CD4 fragments with enhanced gp120 binding ability is as follows: amino acid residues present at the three  
15 sites in human CD4 (as represented in Figure 1) and amino acid residues present at the corresponding positions in the three mutant CD4 molecules are excluded from the group of amino acid residues to be assessed for their effects on gp120 binding ability  
20 when they are incorporated at these sites. Also excluded are amino acids having similar characteristics (e.g., nonpolar R groups, uncharged polar R groups, etc.). Mutants are then produced to include amino acid residues other than those  
25 eliminated from consideration in this manner. Each mutant is then assessed using the anti-CD4 immunoprecipitation and anti-gp120 coprecipitation techniques described.

As a result, modified soluble human CD4  
30 fragments having enhanced gp120 binding ability can be identified. Similar techniques can be used to identify additional critical sites, if such sites exist, and, subsequently, to make substitutions and

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assess their effects on gp120 binding ability of the resulting modified soluble CD4 fragments.

Production of Modified Soluble CD4 Fragments Having Altered gp120 Binding Ability

05        Modified soluble CD4 fragments having altered gp120 binding ability are produced using the techniques described in detail in Examples 4 and 5. Briefly, they are produced as follows:

10        DNA encoding a desired CD4 fragment is produced, either by using recombinant DNA techniques, such as excising it from a vector containing cDNA encoding such a fragment, or by synthesizing DNA encoding the desired fragment mechanically and/or chemically, using known techniques. DNA produced by these  
15        techniques encodes a soluble CD4 fragment which includes none of the hydrophobic transmembrane region of CD4 or a portion of that region (generally six amino acids or less) small enough that it does not prevent solubilization of the fragment. In addition,  
20        particularly in the case of CD4 fragments having enhanced gp120 binding ability, the CD4 fragment is long enough (e.g., 10 amino acids or more) to bind effectively to HIV gp120 envelope protein.

25        Templates for subsequent mutagenesis are produced, using the CD4 fragment-encoding cDNA or DNA. As described below, this can be carried out using a single-stranded bacteriophage cloning vehicle, such as M13. This results in production of single-stranded DNA homologous to only one of the two  
30        strands of the DNA encoding the desired CD4 fragment. The resulting single-stranded DNA is used as a

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template for producing the desired modified soluble CD4 fragments, as follows:

Oligonucleotides are produced, such that their sequence includes a base change or changes which, when incorporated into the nucleotide sequence of DNA subsequently used for the production of CD4 fragments, results in the desired change in the encoded CD4 protein (i.e., different from that encoded by the nucleotide sequence of Figure 1). Such oligonucleotides are produced using standard methods. Oligonucleotides having a base change or base changes are referred to as mutagenized or mutant oligonucleotides.

The mutant oligonucleotide produced in this manner is hybridized to (e.g., by being kinased) the template produced as described above, to produce a template-mutant oligonucleotide complex, referred to as a mutant primer/template. The mutant primer/template is used for the production of a second strand of DNA, using well-known techniques. For example, synthesis of the second DNA strand is carried out by the Klenow fragment of DNA polymerase in the presence of dCTP. Taylor, J.W. et al., Nucleic Acids Research, 13:8749-8764 (1985); Taylor, J.W. et al., Nucleic Acids Research, 13:8764-8785 (1985); Nakayama, K. and F. Eckstein, Nucleic Acids Research, 14:9679-9698 (1986). The resulting strand of DNA contains a modification (or modifications) in the nucleotide sequence of T4 cDNA (i.e., is different from the nucleotide sequence represented in Figure 1) and is referred to as a mutant strand.

Unreplicated single-stranded DNA is removed and the double-stranded DNA is nicked with a selected



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restriction enzyme (e.g., NciI, which does not cut phosphorothioate DNA and, thus, does not cut the new DNA strand containing dCTP S or the mutant strand). Nicked, nonmodified DNA is removed by digestion with  
05 another enzyme, such as exonuclease III. The resulting gapped DNA is repolymerized and, because the mutant strand serves as the template for repolymerization, the mutation or modification is copied into both strands.

10 Once produced, the double-stranded DNA, in which both strands contain the mutation or modification encoding the corresponding modification in the amino acid sequence of the desired soluble CD4 fragment is introduced into a competent host cell, such as a  
15 competent bacterial host (e.g., by transformation). The resulting plaques are grown and DNA contained in them is isolated, using known techniques, and sequenced to confirm the presence of the mutation.

The mutated DNA produced in this manner is  
20 excised from the M13 vector containing it, introduced into a suitable expression vector, such as CDM8, and transfected into an appropriate host cell, such as Cos cells, in which it is expressed. Aruffo, A. and B. Seed, Proceedings of the National Academy of Sciences, USA, 84:3365-3369 (1987). As a result,  
25 mutant CD4 proteins can be assayed, using known techniques. The vector-insert ligation mixture is introduced into competent host bacteria, such as the publicly available E. coli MC1061P3, and  
30 radiolabelled T4 DNA is used to identify CDM8 containing mutant T4 cDNAs.

Production, in Cos cells transfected with the vector containing mutant T4 cDNA, of modified soluble

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CD4 fragments having the desired alteration in gp120 binding ability is subsequently assayed, using known techniques described below.

05 As a result of this procedure, double stranded DNA encoding a modified soluble CD4 fragment having altered gp120 binding ability is produced, the encoded CD4 fragment is expressed and its ability to bind the HIV gp120 envelope protein is assessed.

10 An alternative approach to producing modified soluble human CD4 fragment having altered gp120 binding ability is to use peptide synthesis to make a peptide or polypeptide having the amino acid sequence of such a fragment.

15 The above-described technique was used for producing the 16 mutant CD4 fragments whose sequences are represented in Table 1. Construction of the 16 mutants, transfections, immunoprecipitations and co-precipitations were carried out as described in Example 4. The presence of each mutant was confirmed  
20 by directly sequencing the plasmid DNA used for individual transfections.

#### Use of Soluble Human CD4 Fragments

Soluble human CD4 fragments of the present invention have diagnostic, preventative and therapeutic applications. For example, biologically  
25 active soluble human CD4 fragments can be used for diagnosis, therapy and prevention of infection by HIV.

For example, such fragments can be used therapeutically (in vivo) to treat individuals infected  
30 with HIV. Such fragments can be administered by an

acceptable route (e.g., intravenously, intramuscularly, intraperitoneally, orally), alone or after combination with an acceptable carrier (e.g., saline buffer). They can be administered to inhibit  
05 binding of HIV to T4 lymphocytes and to inhibit HIV transmission from an infected cell to uninfected cells by interfering with syncytium formation. The quantity of such CD4 fragments administered will be determined on an individual basis, but will generally  
10 range from approximately 10 ug/kg body weight to approximately 500 ug/kg body weight per day (in one or more doses per day).

Biologically active soluble CD4 fragments of the present invention can also be used for diagnostic  
15 purposes. For example, they can be used in known immunoassay procedures for detecting the presence and determining the quantity, if desired, of HIV gp120 envelope protein (and, as a result, of HIV itself) in samples, such as blood, semen and saliva. CD4  
20 fragments of the present invention can be, for example, attached or bound by virtue of the CD4 fragment to a solid support, such as latex beads, which are then contacted with a sample to be assayed, in such a manner that if HIV is present in the  
25 sample, it will be bound (by virtue of the CD4 fragment-gp120 interaction). This can be followed by precipitation and/or labelling through contact with an anti-gp120 antibody and detection of the precipitate or labelled product, using known techniques.

30 Biologically active soluble CD4 fragments can also be used for the prevention of HIV infection. For example, such fragments can be incorporated in or attached to materials which might come in contact

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with HIV. They can be incorporated into spermicides, incorporated into or attached to surfaces of condoms, materials from which surgical gloves, dressings and other medical equipment are made or attached to the surfaces of containers or other materials (e.g., filters) for receiving, processing and/or storing blood. In each case, the CD4 fragments of the present invention will bind to HIV gp120 envelope protein (and, thus, to HIV), which will be prevented from further passage (e.g., in the case of spermicides, condoms) or can be removed (e.g., in the case of donated or stored blood).

It is reasonable to assume that the modified soluble CD4 fragments of the present invention with altered (i.e., enhanced gp120 binding ability) will be shown to have the same advantage described for soluble human CD4 fragments. That is, it is reasonable to assume such fragments of the present invention have the capacity to bind the HIV gp120 envelope protein and interfere with HIV infection of T cells, but will not interfere with the function or proliferation of human T lymphocytes which are not infected with HIV. The capability of fragments to bind gp120 envelope protein and interfere with HIV infection and their lack of interference with uninfected T lymphocytes can be assessed by means described herein.

Modified soluble human CD4 fragments having altered gp120 binding ability can be used for therapy, diagnosis and prevention of infection by HIV. For example, use of fragments having slightly reduced or turned down affinity may improve the

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effective pharmacokinetics of therapy. For example, such fragments can be used to bind or hold on to gp120 (and, thus, HIV) transiently. Such fragments bind the virus long enough to render it ineffective as an infectious agent and to prepare it to bind or accept another therapeutic agent (e.g., one which will destroy the virus).

In addition, the region of the CD4 domain I implicated as a possible binding site for gp120 is distinct from the loops analogous to hypervariable complementarity determining segments. If those loops form a binding site for class II MHC, the putative natural ligand of CD4, one can speculate that gp120 may be incapable of inhibiting class II recognition events, even after it has bound to the CD4 structure. Thus, the CD4 mutant described herein should be useful in future analysis of CD4 class II MHC interactions.

Fragments of the present invention having enhanced gp120 binding ability can be used therapeutically (in vivo) to treat individuals infected with HIV. Such fragments can be administered by an acceptable route (e.g., intravenously, intramuscularly, intraperitoneally, orally), alone or after combination with an acceptable carrier (e.g., saline buffer). Modified soluble CD4 fragments with enhanced gp120 binding ability of the present invention can be administered to inhibit binding of HIV to T4 lymphocytes and to inhibit HIV transmission from an infected cell to uninfected cells by interfering with syncytium formation. The quantity of such CD4 fragments administered will be determined on an individual

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basis, but will generally range from approximately 10 ug/kg body weight to approximately 500 ug/kg body weight per day (in one or more doses per day).

05 Modified soluble CD4 fragments having enhanced  
gp120 binding ability can also be used for diagnostic  
purposes. Because of their enhanced binding ability,  
they can be used in known immunoassay procedures for  
detecting the presence and determining the quantity,  
if desired, of HIV gp120 envelope protein (and, as a  
10 result, of HIV itself) in samples, such as blood,  
semen and saliva. CD4 fragments of the present  
invention can be, for example, attached or bound by  
virtue of the CD4 fragment to solid support, such as  
latex beads, which are then contacted with a sample  
15 to be assayed, in such a manner that if HIV is  
present in the sample, it will be bound (by virtue of  
the CD4 fragment-gp120 interaction). This can be  
followed by precipitation and/or labelling through  
contact with an anti-gp120 antibody and detection of  
20 the precipitate or labelled product, using known  
techniques.

Modified soluble CD4 fragments having enhanced  
gp120 binding ability can also be used for the  
prevention of HIV infection. For example, such  
25 fragments can be incorporated in or attached to  
materials which might come in contact with HIV. They  
can be incorporated into spermicides; incorporated  
into or attached to surfaces of condoms, materials  
from which surgical gloves, dressings and other  
30 medical equipment are made; or attached to the  
surfaces of containers or other materials (e.g.,  
filters) for receiving, processing and/or storing  
blood. In each case, the CD4 fragments of the

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present invention will bind to HIV gp120 envelope protein (and, thus, to HIV), which will be prevented from further passage (e.g., in the case of spermicides, condoms, surgical gloves, dressings) or  
05 can be removed (e.g., in the case of donated or stored blood).

#### Example 1 Production of Soluble CD4 Fragments

Initially, cDNA encoding human CD4 was engineered in order to delete the nucleotide sequence  
10 encoding the hydrophobic transmembrane region, which ordinarily renders CD4 membrane bound and insoluble. As a result, cDNA encoding soluble human CD4 fragments was produced.

#### Plasmid Construction

15 Plasmid construction can best be described with reference to Figure 2. As shown in Figure 2, plasmid vector pAc373/T4<sub>ex</sub>, containing the truncated CD4 gene, was constructed from plasmids pAc373 and pSP65-T4.

20 The plasmid transfer vector pAc373 contains a single BamHI cloning site 8 base pairs upstream of the polyhedrin ATG start site. In order to produce a secreted form of the CD4 molecule, the plasmid CD4 protein-encoding pSP65-T4 (kindly provided by Dan  
25 Littman, Univ. of California, San Francisco, CA) was digested with BamHI and XhoI to release the CD4 cDNA insert (which can be readily obtained as described in the literature, as in, for example, Madden *et al.*  
30 *Cell*, 42: 93-104 (1985)). The CD4 cDNA insert was subsequently digested with NciI, which cleaves CD4 cDNA at nucleotide positions 83, 1253 and 1604,

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producing a fragment of 1.17Kb which lacks the ATG start codon and terminates just prior to the trans-membrane region.

Two oligonucleotides, 5'AGGATCCTTAATGAACC3' and  
05 5'CGGTTCATTAAGGATCCT3', were synthesized, using  
standard cyanoethyl phosphoramidite chemistry. They  
were annealed and kinased to generate a linker  
molecule which reconstructs the ATG translation  
initiation codon, includes a stop codon (TAA) for  
10 termination of transcription, creates an NciI  
cohesive end, and adds a BamHI cloning site. Linkers  
were ligated to the 1.17Kb CD4-encoding fragment and  
then digested with BamHI to generate BamHI cohesive  
ends. Subsequently, the CD4-encoding fragment was  
15 inserted into the BamHI cloning site of the publicly  
available transfer vector pAc373. Recombinant  
plasmids containing a single copy of the truncated  
CD4 molecule in the correct orientation were  
identified by restriction mapping. The constructs  
20 were then sequenced by the <sup>35</sup>S-ATP labelled dideoxy  
method to confirm the expected sequence at the  
junctions of insertion. Recombinant plasmids  
pAc373/T4<sub>ex1</sub> and pAc373/T4<sub>ex2</sub> were characterized in  
detail. They contained identical 5' ends. The  
25 synthetic linker ligated in the expected orientation  
in pAc373/T4<sub>ex1</sub> to result in a predicted CD4 protein  
carboxy-terminus of LPTWSTPVH.

Transfer of the T4<sub>ex</sub> sequence from the plasmid  
vector to the Autographa californica nuclear  
30 polyhedrosis virus (AcNPV) genome was accomplished  
essentially as described in Smith et al. (1985)  
P.N.A.S. U.S.A. 82, 8404-8408. In this method,  
cotransfection by calcium phosphate precipitation of



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4 ug pAc373/T4<sub>ex</sub> DNA with 1 ug of purified AcNPV DNA into S. frugiperda cells (SF9), which are publicly available, resulted in homologous recombination between the recombinant sequence of the transfer vector and the polyhedrin gene sequence of AcNPV. Recombinant AcNPV contains an inactivated polyhedrin gene which no longer forms occlusions in infected cells. For plaque purification,  $2 \times 10^5$  SF9 cells were seeded in 100 mM Petri dishes approximately 24 hours prior to assay. Ten fold dilutions of viral supernatant were prepared using final media [Grace's insect medium (Gibco, Grand Island, NY), Difco TC yeastolate 0.33%, lactalbumin hydrolysate 0.33%, 2 mM supplemental glutamine and 50 ug/ml gentamycin containing 10% FCS (Hyclone, Loga, UT)]. Each plate was inoculated with 1 ml of virus ( $10^{-2}$  to  $10^{-7}$  dilution) plus 2 ml of final media. After incubation for 2 hours, the inoculum was removed and replaced with 10 ml of 1.5% Sea Plaque agarose (FMC Bioproducts, Rockland, ME) in final media. After agarose solidification, plates were transferred to a humid environment for 4-6 days at 27°C.

Plaque assay of the transfection supernatant yielded plaques of distinct morphology: either infected cells which are occlusion positive (wild type AcNPV) or occlusion negative (recombinant CD4 virus). Occlusion-negative plaques were identified, selected, and further plaque purified. DNA from cells infected with putative CD4 recombinant virus was hybridized with a  $^{32}$ P labelled CD4 cDNA probe to verify the presence of the CD4 sequence. Production of the T4<sub>ex</sub> polypeptide was carried out as follows:  $6 \times 10^5$  SF9 cells were seeded per well in 24 well

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Nunc plates (Interlab, Thousand Oaks, CA) for 2 hours at 27°C and then adherent cells infected with virus at an MOI of 10 in 0.2 ml final media for 2 h. The innoculum was then removed and cells cultured in 0.5 ml fresh medium at 27°C for 48 hours. Adherent cells were then washed twice with 0.5 ml Grace's medium lacking serum and methionine followed by incubation in 0.5 ml in the same medium for 1 hour. The adherent cells were washed once and then cultured for 6 hours in serum and methionine-free Grace's medium containing 67 uCi <sup>35</sup>S methionine (New England Nuclear, Boston, MA 1134 Ci/mmol). Culture supernatants were harvested, microfuged for 10 minutes, and dialyzed at 4°C against PBS containing 0.05% sodium azide and 10 mM cold methionine. Cells were dislodged from the wells, washed twice with Grace's medium at 4°C (by centrifugation in a Sorvall RT6000 for 5 minutes at 1000 rpm) and finally lysed for 30 minutes at 4°C by the addition of a RIPA buffer containing 1% Triton X-100, 0.15 M NaCl and a cocktail of protease inhibitors (see below).

The lysates were microfuged for 10 minutes and dialyzed at 4°C as for culture supernatants. Both lysates and culture supernatants were subjected to immunoprecipitation for 16 hours at 4°C with a monoclonal anti-CD4 antibody (19Thy5D7) linked to Affigel-10 beads (5 mg monoclonal antibody/ml gel). After immunoabsorption, the beads were washed twice with lysis buffer and bound material was eluted by treatment of the beads with 0.1 M glycine-HCl buffer, pH 2.0. Eluates and whole samples of lysates or culture supernatants were mixed with SDS sample buffer containing 2-mercaptoethanol, boiled for 5

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minutes and electrophoresed in 12.5% mini-slab gels according to Laemmli. Subsequently, the gels were fixed, dried and autoradiographed using Kodak XAR-5 film. High titer viral stocks were generated by  
05 infecting SF9 cells at an MOI of 1 and culturing at  $1 \times 10^6$  cells/ml for 4 days in final media. These stocks were used for infecting SF9 cells for production of protein. For large scale production of  
10 protein, SF9 cells were grown in 2 liter spinner flasks in final media. Cells were harvested and infected with an MOI of 15 (using high titer viral stocks) at a concentration of  $10 \times 10^6$  cells/ml. Cells were then pelleted, resuspended in media at  
15  $1 \times 10^6$ /ml, and cultured for 3 days at 27°C in spinner flasks. At this time, supernatants were collected by centrifuging cultures to remove cells.

For large scale purification, infected SF9 cell culture supernatants were harvested by centrifugation of cells in a Sorvall H-4000 rotor at 800 rpm for 6  
20 minutes at 4°C. The culture supernatants were then subjected to protease inhibition by the addition of a cocktail of protease inhibitors made up of leupeptin, antipain, pepstatin, and chymostatin to final concentrations of 0.5 ug/ml; soybean trypsin inhibitor to  
25 0.02 ug/ml; and phenyl methyl sulfonyl fluoride (PMSF) to 1.25 mM, followed by adjustment of the pH to 6.8 by the dropwise addition of 1 M NaOH. The samples were subsequently clarified by centrifugation in a Sorvall GSA rotor at 8000 rpm for 25 minutes at  
30 4°C and pumped at 4°C at a flow-rate of 30 ml/hour through a 2 ml precleared immunoabsorbent column, 21Thy2D3 monoclonal antibody (anti-T8) coupled to Affigel-10 (Biorad), followed in series by a 7 ml

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column of anti-CD4 monoclonal antibody (19Thy5D7) coupled to Affigel 10 at a concentration of 7.5 mg monoclonal antibody per ml of gel; the monoclonal antibodies were made according to conventional methods. The anti-CD4 column was then washed with 30 ml of 10 mM Tris-HCl buffer, pH 5.0. The bound CD4 polypeptides were eluted by pumping 0.1 M glycine-HCl, pH 2.0, through the washed anti-CD4 column and 0.8 ml fractions of eluant were collected into tubes containing 0.15 ml 1 M Tris-HCl, pH 7.6. During the whole column fractionation procedure, eluate absorption was monitored at 280 nm with a Uvicord 2 (LKB, Gaithersburg, MD) fitted with an event marker. Fractions of neutralized pH 2.0 eluate containing protein were pooled and concentrated by ultrafiltration in a stirred cell (Amicon, model 3) fitted with a YM-5 membrane. Typically, the yield of purified T4<sub>ex</sub> polypeptides was 1 ug/ml of infected SF9 culture supernatants. Aliquots containing 1 ug of protein concentrate (assuming that 1 OD unit = 1 mg/ml at a 280 nm) were examined for purity in 12.5% SDS-polyacrylamide slab gels, stained with Coomassie blue.

Purification and characterization of the T4<sub>ex</sub> polypeptides

SF9 cells infected with either recombinant baculovirus containing the T4<sub>ex</sub> cDNAs or wild type AcNPV virus were cultured in <sup>35</sup>S-methionine and products were examined by SDS-PAGE, followed by autoradiography.

It was shown that the T4<sub>ex1</sub> polypeptide is the major secreted product of SF9 cells infected with the

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T4<sub>ex1</sub> recombinant baculovirus. The predominant <sup>35</sup>S labelled protein band (45% of total labelled material) in SDS-PAGE analysis of supernatants from SF9 cells obtained 54 hours after T4<sub>ex1</sub> recombinant baculoviral infection is a 50KD band under reducing conditions. This band co-migrates with material immunoprecipitated by anti-CD4 monoclonal antibody (19Thy5D7) from T4<sub>ex1</sub> baculovirus infected SF9 supernatants or cell lysates. In addition, the latter shows a strongly labelled band of 52KD which presumably represents the T4<sub>ex1</sub> polypeptide still carrying the uncleaved signal peptide. Although a 50KD band is readily detected in the total cell lysate of T4<sub>ex1</sub> virus infected cells even in the absence of immunoprecipitation with anti-CD4 monoclonal antibody, it is a minor component of a complex mixture of labelled intracellular polypeptides. As expected, no CD4 material was precipitated from supernatants of wild type AcNPV-infected cells or detectable in the total supernatant.

Each of two representative T4<sub>ex2</sub> preparations yielded a protein that migrated under reducing conditions with a molecular weight of 51KD (and is glycosylated as indicated by endoglycosidase F experiments), whereas the T4<sub>ex1</sub> protein migrated slightly faster with a molecular weight of 50KD. These different mobilities in SDS-PAGE between T4<sub>ex1</sub> and T4<sub>ex2</sub> proteins are not unexpected, since T4<sub>ex2</sub> contains 17 additional carboxy terminal amino acids derived from fusion with the polyhedrin gene. Under nonreducing conditions, the mobility of T4<sub>ex1</sub> protein is faster than under reducing conditions, consistent

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with previous predictions that there are intrachain disulfide bonds in the CD4 external segment, and also showing the absence of covalent disulfide linked polymers of T4<sub>ex1</sub> protein. The protein production strategy described above routinely yields 1-2 mg of secreted T4<sub>ex1</sub> or T4<sub>ex2</sub> proteins per liter of SF9 cells (1-2 x 10<sup>9</sup> cells) over a 72 hour culture period.

To verify that the 50KD T4<sub>ex1</sub> and 51KD T4<sub>ex2</sub> proteins were indeed the products of the CD4 gene, purified polypeptides were electroblotted onto polyvinylidene difluoride membranes (Millipore, 0.45 µm pore size) and the Coomassie blue stained 50-51KD material subjected to amino terminal sequencing on an Applied Biosystems model 470A sequenator equipped with an on-line 120A PTH analyzer using the 03R PTH program. In each case, the first 10 cycles yielded the unambiguous sequence: KKVVLGKKGD. In contrast, the predicted N-terminal sequence of CD4 based on translation of the cDNA nucleotide sequence previously has been suggested to be either QGNKVVLGKKGD or NKVVLGKKGD. The second of these two assignments was based on homology with the rat N-terminal sequence RTVVLGK. While the empirically derived sequences herein are consistent with the positioning of latter N-terminal human assignment and that of CD4 in mouse and sheep, the K at position 1 assigned by the present amino acid sequence analysis is at variance with the amino acid predicted from the nucleotide sequence of the cDNA. To resolve these differences, DNA sequencing of pAc373/T4<sub>ex2</sub> inserts was carried out. The codon for the amino terminal residue was determined to be AAG, rather than AAC, as given in

the original CD4 cDNA cloning paper (Madden et al.,  
id) without other differences noted. Whether this  
single nucleotide discrepancy represents a mutation  
resulting from cloning into pAc373 is not known, but  
05 appears unlikely in view of the lysine residue found  
at the N-terminus of the homologous rat CD4 sequence.  
From these data, it was concluded that the amino  
terminus of mature human CD4 begins with two lysine  
residues, followed by two valine residues and that  
10 T4<sub>ex1</sub> and T4<sub>ex2</sub> are CD4 derived polypeptides. In  
addition, this data shows that the baculovirus  
expression system has the capacity to enzymatically  
cleave the signal peptide from the T4<sub>ex</sub> polypeptide  
precursor, allowing it to be secreted. Thus, the  
15 hydrophobic transmembrane portion of the CD4 protein,  
which ordinarily causes the protein to be insoluble,  
is deleted, as are the first three or four external  
amino acids adjacent the transmembrane portion. This  
means that the truncated soluble CD4 polypeptides  
20 have 371 amino acid residues (T4<sub>ex1</sub>) or 370 amino  
acid residues (T4<sub>ex2</sub>), compared to the 374 amino acid  
mature extracellular segment.

#### Binding of Soluble CD4 Fragments to HIV gp120

To determine whether the soluble CD4 proteins  
25 produced in the baculovirus system could bind to the  
HIV gp120 exterior glycoprotein, the following two  
reciprocal coprecipitation experiments were carried  
out. First, metabolically labelled gp120 protein  
derived from HIV virions was incubated with un-  
30 labelled purified T4<sub>ex2</sub> either in the absence or  
presence of monoclonal antibodies directed against  
distinct epitopes of the CD4 protein (OKT4 and

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OKT4A). The OKT4A antibody (like 19Thy5D7), but not the OKT4 monoclonal antibody, is known to inhibit the binding of gp120 to the CD4 molecule. Culture supernatants were collected from  $1 \times 10^7$  Molt-3 lymphocytes stably infected with the HIV strain IIIB that were metabolically labelled overnight with 100 uCi/ml.  $^{35}\text{S}$ -cysteine in total volume of 1.5 ml. NP-40 was added to a final concentration of 0.5% and the supernatants were incubated with 10 ug of  $\text{T4}_{\text{ex2}}$  for 1 hour at 37°C, with or without preincubation of the soluble T4 with 5 ug/ml of OKT4A (Ortho Pharmaceutical, Raritan, NJ). The samples were then immunoprecipitated with the monoclonal antibody OKT4 and run on SDS polyacrylamide gels as described in Kowalski et al., Science, 237:1351-1355 (1987). In addition, 1.5 ml of unlabelled culture supernatants were collected from either  $1 \times 10^6$  uninfected Molt-3 lymphocytes or HIV-infected Molt-3 lymphocytes and incubated for 1 hour at 37°C with  $^{125}\text{I}$  labelled soluble T4 that had been radioiodinated by Bolton-Hunter reagent (NEN, Boston, MA). The samples were then immunoprecipitated using a goat anti-gp120 antiserum, as described in Kowalski et al., id.

In the absence of OKT4A monoclonal antibody preincubation, the gp120 protein was coprecipitated by OKT4 monoclonal antibody. In contrast, preincubation with OKT4A antibody inhibits gp120 co-precipitation. These findings show that gp120 binds to the  $\text{T4}_{\text{ex2}}$  protein and that this binding is inhibited by OKT4A. The OKT4 monoclonal antibody did not precipitate the gp120 protein in the absence of added  $\text{T4}_{\text{ex2}}$  protein.



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In a second experiment, unlabelled HIV virions were incubated with radioiodinated T4<sub>ex2</sub> protein. The mixture was then immunoprecipitated with a goat antiserum raised against purified HIV gp120 protein.

05 The iodinated T4<sub>ex2</sub> protein was coprecipitated by the anti-gp120 serum only when HIV virions were present, indicating that the T4<sub>2x2</sub> protein was capable of binding to an HIV virion component.

#### Inhibition of gp120 Binding to T4 Soluble CD4

##### 10 Fragments

To examine whether the T4<sub>ex1</sub> protein could inhibit the binding of gp120 protein to CD4<sup>+</sup> lymphocytes, metabolically labelled gp120 protein from the supernatants of virus-infected cells was

15 preincubated with T4<sub>ex1</sub> protein or a control protein made in the baculovirus system (an extracellular T11 segment), as follows.

1 x 10<sup>7</sup> H9 lymphocytes stably infected with the HIV strain IIIB were metabolically labelled with

20 <sup>35</sup>S-cysteine overnight in a total volume of 1.5 ml. The supernatants containing labelled HIV proteins were incubated for 1 hour at 37°C with either: phosphate buffer saline (PBS), 2.5 ug/ml OKT4A; 30 ug/ml T4<sub>ex1</sub>; or 30 ug/ml T11. The SupT1 cells were

25 centrifuged, washed once with PBS, lysed with 0.75 ml of lysis buffer and the gp120 bound to the SupT1 cells was immunoprecipitated as described in Kowalski et al., *id.* The OKT4 and OKT4A monoclonal antibodies were added to the SupT1 cells prior to the addition

30 of the labelled protein to control for specificity of the binding.

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The OKT4A but not the OKT4 monoclonal antibody was found to inhibit the binding of the labelled gp120 protein to the SupT1 cells. The T4<sub>ex1</sub> protein significantly inhibited the binding of labelled gp120 to the surface of SupT1 cells. No inhibition was observed using up to a 30 ug/ml concentration of the control protein, whereas inhibition of gp120 binding was seen at 0.5 ug/ml concentration of T4<sub>ex1</sub> protein.

#### Effect of Soluble CD4 Fragments on HIV Replication

To determine whether the T4<sub>ex1</sub> polypeptide would inhibit infection of cells by HIV, experiments were carried out using two types of lymphocytes (C8166 and H9). For each cell type,  $2 \times 10^5$  cells were infected with 1000 TCID<sub>50</sub> units of the H9III<sub>2</sub> strain of HIV, in the presence of proteins, which were added at the time of infection and maintained at the following concentrations throughout the course of the experiment: no added protein; ovalbumin (control), 10 ug/ml; T4<sub>ex2</sub> protein, 10 ug/ml; T4<sub>ex2</sub> protein, 2 ug/ml; T4<sub>ex2</sub> protein, 0.2 ug/ml; 21 Thy2D3 (anti-CD8, control), 10 ug/ml; and 19Thy5D7 (anti-CD4, control), 0.2 ug/ml. On day 9 following infection, supernatants were collected and assayed for viral p24 gag protein by radioimmunoassay. Referring to Figure 4, the amount of p24 protein in cell supernatants is plotted vs. the concentration of added ovalbumin (□), soluble T11 produced in baculovirus (■), T4<sub>ex1</sub> protein (○), T4<sub>ex2</sub> protein (●) or 19Thy5D7 ( ).

As shown in Figure 4, while ovalbumin, recombinant T11 and 21 Thy2D3 proteins exerted no effect on virus replication, ug/ml concentrations of

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T4<sub>ex1</sub> and T4<sub>ex2</sub> and anti-T4 (19Thy5D7) exhibited significant inhibition of viral protein expression and virus production. The T4<sub>ex1</sub> and T4<sub>ex2</sub> proteins were able to decrease HIV p24 protein expression at concentrations of 0.2 ug/ml. These studies indicate that the T4<sub>ex1</sub> and T4<sub>ex2</sub> proteins inhibit HIV replication in CD4+ lymphocytes.

Inhibition of HIV Envelope-induced Syncytia by Soluble CD4 Fragments

The induction of syncytia by the HIV envelope depends upon binding of the gp120 exterior glycoprotein to the CD4 molecule, followed by events involved in membrane fusion. To examine whether the T4<sub>ex2</sub> protein could inhibit the formation of syncytia by the HIV envelope, cells chronically infected with HIV were cocultivated with CD4+ SupT1 lymphocytes in the presence or absence of the T4<sub>ex2</sub> protein. Addition of control proteins, ovalbumin, or an anti-T8 monoclonal antibody (21Thy2D3) to the cocultivated cells had no effect on the formation of syncytia, which were scored at 6 h after the cocultivation had begun (Table 2). By contrast, addition of as little as 2 ug/ml of T4<sub>ex2</sub> or anti-T4 (19Thy5D7) was able to completely inhibit the formation of syncytia in this assay. Both T4<sub>ex1</sub> and T4<sub>ex2</sub> proteins inhibited the induction of syncytia when CHO cells constitutively expressing the HIV envelope were cocultivated with SupT1 lymphocytes. No inhibition of syncytium formation was observed with a recombinant secreted T11 protein made in a baculovirus expression system.

TABLE 2

## INHIBITION OF HIV ENVELOPE-INDUCED SYNCYTIA BY SOLUBLE T4 PROTEINS

	<u>Protein</u>	<u>Concentration (<math>\mu</math>g/ml)</u>	<u>Syncytia</u>
	None	-	—
05	Ovalbumin	50	—
		20	—
		5	—
		2	—
		50	—
10	Anti-T3 (21Thy2D3)	20	—
		5	—
		2	—
		50	-
		20	-
15	Anti-T4 (19Thy5D7)	5	-
		2	-
		50	-
		20	-
		5	-
20	T4ex2 protein	2	-
		50	-
		20	-
		5	-
		2	-

Approximately  $5 \times 10^5$  SupT1 CD4+ lymphocytes were cocultivated with  $1 \times 10^4$  Jurkat lymphocytes infected with the HXBol strain of HIV in 1 ml volume in the presence of the indicated concentrations of protein. Ten hours after beginning of cocultivation, dilutions were made and number of syncytia per milliliter estimated (—, >1000; +, 500-1000; ++, 50-500; -, <50).

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To assess whether the observed inhibition of syncytia by the T4<sub>ex2</sub> product was due to its interaction with the envelope-expressing cells or with the CD4+ "target" cells, either of these cells was

05 separately incubated with the T4<sub>ex2</sub> protein, washed, and then used in the cocultivation assay. Table 3

shows that the pretreatment of the envelope-expressing cells (H9/HTLVIII2) was as effective at syncytia inhibition as was pretreatment of both

10 envelope-expressing and CD4+ (SupT1) cells. In contrast, incubation of the "target" SupT1 cells with the T4<sub>ex2</sub> protein exhibited only slight effects on syncytium formation. Thus, the soluble CD4 fragments appears to exert their syncytium-inhibiting effect

15 through its interaction with the envelope-expressing cells.

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TABLE 3

INHIBITION OF SYNCYTIIUM FORMATION BY INTERACTION  
WITH ENVELOPE-EXPRESSING CELLS

	Number of syncytia	
	SupT1 + ovalbumin	SupT1 + T <sub>4</sub> ex2
H9/HTLV-III <sub>B</sub> + ovalbumin	1560	1100
H9/HTLV-III <sub>B</sub> + T <sub>4</sub> ex2	110	110

Approximately  $1 \times 10^6$  H9/HTLV-III<sub>B</sub> lymphocytes or  $4 \times 10^6$  SupT1 lymphocytes were incubated in medium with either ovalbumin or T<sub>4</sub>ex2 protein at a concentration of 20  $\mu$ g/ml at 37°C for 30 min. The cells were then centrifuged and washed with phosphate buffered saline, centrifuged and resuspended in medium. The treated H9/HTLV-III<sub>B</sub> were then mixed with the treated SupT1 cells in 24 well dishes and returned to a 37°C, 5% CO<sub>2</sub> incubator for 5 h, when total syncytia per well were counted.

Lack of Inhibition of Class II MHC Recognition Events  
by Soluble CD4 Fragments

Because CD4 function is necessary for facilitating activation of class II specific CTL and Ia restricted helper T lymphocytes, experiments were carried out to examine whether the same  $T4_{ex}$  proteins abrogate physiologic response of CD4+ T lymphocytes. In this regard, two types of experiments were performed. The first examined the effect of  $T4_{ex2}$  on class II MHC directed killing mediated by the CD4+ cytolytic clone AA8. The  $T4_{ex2}$  cytolytic clone AA8 is specific for class II MHC gene products on the allogeneic EBV transformed B cell line Lax 509. Referring to Figure 3,  $^{51}Cr$  labelled Lax 509 cells were preincubated with secreted  $T4_{ex2}$  ( $\Delta$ ) or a control protein (BSA ( $\square$ )) for 30 min at 4°C prior to addition of effector cells. In other wells, anti- $T4$  (19Thy5D7) ( $\circ$ ) and anti-CALLA J5 (kind gift of Jerome Ritz, Dana Farber Cancer Institute, Boston, MA) ( $\bullet$ ) antibodies were used as inhibitors. After preincubation of targets (3000 cells/well) with secreted CD4 fragment inhibitors, lysis was measured in a standard 4h  $^{51}Cr$  release assay at an E/T ratio of 30:1. Results shown are the mean of quadruplicate samples where standard deviations are greater than 10%.

As shown,  $T4_{ex2}$ , like the control protein BSA and the anti-T8 monoclonal antibody, failed to inhibit CTL effector function even at concentrations as high as 100 ug/ml. In contrast, as little as 1-3 ug/ml of specific anti- $T4$  (19Thy5D7) monoclonal antibody reduced cytotoxicity by less than 50%.  $T4_{ex1}$  also was without effect on cell lysis.

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Lack of Effect on T-Cell Proliferation by Soluble CD4  
Fragments

The following experiment was carried out to determine the effect of T4<sub>ex1</sub> on proliferation of the  
05 T4+ tetanus toxoid specific, class II MHC restricted  
helper T cell clone CTT7. Referring to Figure 6, for  
proliferative studies, 50,000 cells/well of the  
tetanus toxoid specific clone CTT7, derived by  
standard cloning strategies, was cultured in 10%  
10 FCS/RPMI 1640 supplemented with 1% pen-strep and 2%  
glutamine alone, with 10 ug/ml of tetanus toxoid  
(Massachusetts Department of Public Health, Jamaica  
Plain, MA) or the combination of tetanus toxoid (TT)  
and 10% autologous macrophages (MO) in the presence  
15 or absence of a final concentration of 40 ug/ml  
ovalbumin (ova), anti-T4, anti-T8, or twice  
immunoabsorbed T4<sub>ex1</sub> for 24 h at 37°C in a humid  
atmosphere with 6% CO<sub>2</sub>. Subsequently, wells were  
pulsed with 1 uCi/well of <sup>3</sup>H-TdR. Cells were har-  
20 vested at 48 h using an automated cell harvester.  
Plus signs (+) indicate presence of a given additive.

As shown in Figure 6, the CTT7 clone is  
activated to undergo proliferation only in the  
presence of tetanus toxoid and the autologous antigen  
25 presenting cell. At a concentration of 40 ug/ml, the  
anti-CD4 (19Thy5D7) monoclonal antibody inhibited  
<sup>3</sup>H-TdR incorporation by 80%, consistent with the  
important role of CD4 in helper T cell response. In  
contrast, equivalent amounts of T4<sub>ex1</sub>, ovalbumin, or  
30 anti-T8 monoclonal antibody have no effects.

Thus, while T4<sub>ex</sub> protein binds HIV gp120 and  
thereby inhibits binding of gp120 to its receptor,  
HIV envelope-induced syncytium formation and HIV



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replication, it has no discernable effect on class II directed physiologic T cell response at identical concentrations under these experimental conditions. The basis for this difference remains to be resolved.

05 One possibility is that the affinity of CD4 for gp120 is substantially higher than CD4 for its native ligand (presumably class II MHC). In addition, because CD4 is only one of several elements (others including LFA-1, T11, etc.) that facilitate cell-cell

10 interactions between CTL and targets or inducer T cells and antigen presenting cells, partial abrogation of the CD4 function with T<sub>4</sub><sup>ex1</sup> protein may still leave the T cell activation process uninhibited.

Thus, concentrations of soluble CD4 fragments in

15 the picomolar range, like certain anti-T4 monoclonal antibodies, inhibit syncytium formation and HIV infection. However, in contrast to anti-T4, the effects of soluble CD4 protein are exerted at the level of gp120 expressing cells. In addition, class

20 II specific T cell interactions are functionally unimpeded by soluble CD4 protein, whereas they are virtually abrogated by equivalent amounts of anti-T4 antibody under the same experimental conditions. Whether this selective effect is a consequence of

25 substantial differences in CD4 affinity for gp120 compared to antibody remains to be determined.

Nevertheless, the present findings indicate that the extracellular segment of the CD4 protein or peptide fragments derived from it can be useful in competi-

30 tively inhibiting the interaction between the native transmembrane CD4 structure on T lymphocytes and the viral gp120 protein. Furthermore, these soluble CD4 proteins should allow the establishment of assays

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designed to detect drugs which might interfere with gp120-CD4 interactions. Importantly, T4<sub>ex</sub> proteins themselves or fragments derived from T4<sub>ex</sub> may have clinical utility in inhibiting gp120 binding to membrane bound CD4 on T lymphocytes, monocytes, or brain cells without interfering with the normal physiological role of surface CD4 on healthy cells.

Example 2 Assessment of Interaction of T4<sub>ex</sub> Proteins and Derived Peptide Fragments with HIV

gp120

To further analyze the specific physical interaction between T4<sub>ex</sub> proteins or their derived peptide fragments and gp120, a method was employed which involved size fractionation by SDS-PAGE followed by electroblotting of the T4<sub>ex2</sub> protein onto polyvinylidene difluoride membranes. T4<sub>ex2</sub> (75 ug in neutralized immunoaffinity eluate) was mixed with a 1/9 volume of 0.9 M Tris-HCL, pH 8.0 containing 40 mM CaCl<sub>2</sub>. TPCK trypsin (Worthington) was added to an enzyme:protein ratio of 1:50 (w/w) and digestion was carried out at 37°C. 25 ug aliquots were removed at 10, 20 and 45 minutes. Digestion was stopped by the addition of a non-reduced SDS sample buffer and heating to 100°C for 5 minutes. Aliquots were electrophoresed on 12.5% mini slab gel under non-reduced conditions. Gels were subsequently electroblotted, using the method of Matsudaira, onto polyvinylidene difluoride membrane (Millipore; 0.45 um pore size). Matsudaira, P., J. Biol. Chem., 262: 10035 (1987). Duplicate tracks were either stained with Coomassie blue or blocked with a 5% dried milk solution in PBS/azide for 2

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hours at room temp. Electrobloods after blocking were assembled in a miniblotted apparatus (Immunetics) and slots overlying the appropriate tracks filled with 50 ul of purified, native HIV gp120 at 20ug/ml. in 1% dried milk in PBS for testing for HIV gp120 reactivity. Incubation with shaking was carried out overnight at 4°C. Following three 5 minute washes with PBS/0.05% Triton X-100, the blots were incubated with radioiodinated mouse monoclonal IgG, anti-HTLVIII gp120 2.6 ug (specific activity, 2uCi/ug) diluted in 25 ml 1% milk in PBS for 1 hour at room temperature, then 1 hour at 4°C.

After five further washes with PBS/Triton X-100 and two washes of PBS the blots were air dried and autoradiographed at -70°C (using preflashed Kodack XAR film and an enhancer screen). Where appropriate, stained bands of interest were cut out and sequenced on a gas phase protein sequencer (Applied Biosystem 470A) with on line PTH analyzer (120A) using the 03RPTH programs.

The single band of T<sub>4</sub><sub>ex2</sub> at 50KD MW when electrophoresed unreduced was shown to be capable of binding HIV gp120 strongly. In contrast, identical amounts of T<sub>4</sub><sub>ex2</sub>, either reduced or reduced and amidomethylated, did not bind gp120 when similarly examined. The lack of gp120 binding to reduced and alkylated T<sub>4</sub><sub>ex2</sub> is not due to the modification during alkylation of the cysteine residues themselves, as shown by the concurrent lack of gp120 binding to reduced T<sub>4</sub><sub>ex2</sub>. The fact that the electrophoretic mobility of the T<sub>4</sub><sub>ex2</sub> protein after reduction is slower than when not reduced is consistent with the prediction that there are intrachain disulfide bonds

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in the external segment of human T4. In addition, the migration of the non-reduced T4<sub>ex2</sub> protein as a single moiety of 50KD shows that the purified protein does not contain disulfide-linked polymers of T4<sub>ex2</sub> protein. Identical results to the above were obtained when using T4<sub>ex1</sub> proteins. Taken together, these results demonstrated that under these conditions, it is likely that the binding of gp120 to T4<sub>ex</sub> proteins is dependent on the presence of intact disulfide bridges within the T4<sub>ex</sub> protein, which are presumably stabilizing the tertiary structure of their binding region.

Enzymic fragmentations on the purified T4<sub>ex</sub> protein carried out as described above produced a wide range of fragments. HIV gp120 binding analysis of blotted material from a 45 minute papain digest demonstrates that, in addition to the expected binding by the 50KD residual T4<sub>ex1</sub> protein, a fragment is present with a mobility of 28KD which binds gp120. In order to definitively identify and purify the 28KD fragment, 40-fold more T4<sub>ex1</sub> protein was digested with papain and separated by preparative SDS-PAGE. A portion of the blot was subjected to analysis for gp120 binding, and comparison of densitometric scans of the stained blot and the autoradiograph showed that the 28 kD material bound a relative amount of gp120 similar to that bound by the residual T4<sub>ex1</sub> protein in the same track. Thus, the 28KD fragment bound HIV gp 120 with the same efficiency as the parent T4<sub>ex2</sub> protein. The 28 KD band was excised from the Coomassie blue stained portion of this same blot and subjected to amino terminal microsequencing. The first 11 cycles

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yielded a single unambiguous sequence of KKVVLGKKGDT, showing that the 28KD fragment is an intact polypeptide chain derived from the amino terminal region of the T4<sub>ex1</sub> protein. Assuming an average MW for each amino acid of 110 daltons, the papain cleavage of T4<sub>ex1</sub> yielding the 28KD fragment can be located as being C-terminal to the cysteine residue at position 159 and with domain 3 proximal to the oligosaccharide addition sites at positions 256 and 300 (Figure 7C). Thus, the binding of HIV gp120 to T4<sub>ex1</sub> does not involve the C-terminal stretch of amino acids in domain 3 containing both N-linked glycosylation sites of the T4 structure, or domain 4. This result defines the gp120 binding portion of T4 as being in the N-terminal region of the protein.

Similar experiments utilized trypsin fragmentation of T4<sub>ex</sub> to further define the nature of the gp120 binding fragments. Digestion of T4<sub>ex2</sub> protein with trypsin produces a set of fragments different from those seen with papain digestion. Analysis of separated material from the 45 minute tryptic digest for gp120 binding shows only a weak signal produced by the trace amount of 50KD T4<sub>ex2</sub> protein left after digestion. Amino terminal sequencing of the strongly Coomassie staining 45 KD heterogeneous band derived from blots of the 45 minute digest shows the presence of two major sequences corresponding to tryptic cleavage at lysine residues 7 and 75 and a minor sequence corresponding to a cleavage at lysine 72 (Fig. 7c). No other signals are seen in this material, indicating that these three lysine residues are highly labile towards trypsin and that such cleavage and/or loss or

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residues 1-7 is sufficient to abrogate the binding of gp120.

05 In order to investigate the possibility that a  
gp120 binding tryptic T4<sub>ex2</sub> fragment could be  
obtained using a more restricted digestion, the  
material migrating in the 40-45 KD region from a 20  
minute tryptic digest of T4<sub>ex2</sub> was examined, and no  
binding ff gp120 was apparent. Microsequencing of  
the 45KD material derived from the 10 minute tryptic  
10 digest gave a mixed sequence, with two major signals  
present, one corresponding to cleavage at lysine 72.  
In addition, there were two minor signals, one  
derived from cleavage at lysine 7 and the other to  
cleavage at lysine 75. This information leads to the  
15 conclusion that probably the most labile tryptic  
residue in T4<sub>ex</sub> is the lysine at 72, and that  
residues 1-7 are not alone responsible for HIV gp120  
binding to CD4.

20 That the only detectable alterations of T4<sub>ex</sub>  
protein after trypsin cleavage are in domain 1, and  
these perturbations are capable of inhibiting gp120  
binding, argues strongly for an essential role of the  
native domain 1 region. The ability of the NH<sub>2</sub>  
-terminal 28KD papain fragment of T4<sub>ex1</sub> to bind HIV  
25 gp120 is consistent with this view. Given the  
requirement of an intrachain disulfide bond to  
maintain the native conformation of Ig-like domains,  
the loss of HIV gp120 binding after T4<sub>ex1</sub> or T4<sub>ex2</sub>  
reduction further supports the notion.

30 Further binding site information is provided by  
the observation that the same restricted tryptic  
cleavage is sufficient to destabilize the antigenic  
epitope recognized by anti-T4 monoclonal antibody

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19Thy5D7. Thus, when tryptic digests of both T<sub>4</sub><sub>ex</sub> proteins are passed through a 19Thy5D7 immunoabsorbant, only the residual intact T<sub>4</sub><sub>ex</sub> protein is bound. The fact that 19Thy5d7 is known to inhibit HIV gp120 binding to CD4 makes this result not unexpected and suggests that the HIV gp120 binding region and the 19Thy5D7 epitope localize to the immunoglobulin V-like NH<sub>2</sub>-terminal segment of CD4. However, given the large size of the antibody molecule relative to T4 domain 1 and, thus, the potential for steric blockage, the location of the 19Thy5D7 epitope relative to the gp120 site cannot be accurately known.

Conservation of cysteines and multiple other invariant Ig residues in the T4 sequence, as well as secondary structure predictions, argue that the first 92 residues of the T4 molecule have similar tertiary structure to an Ig V domain. It thus could be expected that the lysine residues at positions 7, 72 and 75 are clustered in space near each other, extending from the surface of the domain. In the case of lysine residues 72 and 75, which fall within a highly conserved area of sequence bounded by a aspartate residue (Figure 7C, amino acid 78) and arginine residue (Figure 7C, amino acid 54) that probably form a salt bridge characteristic of V domains, this alignment is almost certainly correct. This cluster may be involved in the binding of HIV gp120 to the CD4 molecule.

The above results indicate that a linear stretch of amino acids is unlikely to be an effective, high affinity inhibitor of CD4-HIV gp120 interaction; the results show that disulfide bridging of T<sub>4</sub><sub>ex</sub> protein

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plays a key role in such interaction. The finding that cleavage at lysine residue 72 (i.e., between the cysteine residues in the V-like domain) is sufficient to destabilize both the gp120 and 19Thy5D7 binding region is consistent with this view. In addition, although these results implicate the V-like domain in the binding of gp120 to T4, the possibility has not as yet been ruled out that domain 2 containing cysteine residues at positions 130 and 159 (Figure 7C) might play a part in conjunction with the V-like domain 1.

Example 3 Production of Biologically Active,  
Modified Soluble Human CD4 Fragments and  
Assessment of Their Activity

15 Modifications of the T4 cDNA were produced using an M13 T4 template. The T4<sub>ex</sub> cDNA fragment was excised from plasmid vector pAc373/T4<sub>ex</sub> using BamHI. The plasmid vector pAc373/T4<sub>ex</sub> is described in Example 1 and in Hussey, *et al.*, *Nature*, 331:78  
20 (1988). The ends of the fragment were blunted with DNA polymerase I and the fragment ligated to XbaI linkers (New England Biolabs). The linkered fragment was digested with XbaI, gel purified to remove excess linkers and ligated to Xba cut M13 replicative form.  
25 The ligation mixture was transformed into competent TG1 host bacteria, plated out and the resulting plaques were screened by hybridization to T4 oligonucleotides. Plaques hybridizing to sense oligonucleotides were grown up to produce  
30 single-stranded M13 templates for mutagenesis.

The mutagenesis protocol is that marketed by Amersham and is based on the method of Eckstein



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(Taylor, et al., Nucleic Acids Research, 13:8749 (1985); Taylor, et al., Nucleic Acids Research, 13:8764 (1985); Nakayama and Eckstein, Nucleic Acids Research, 14:9679 (1986)). Oligonucleotides were produced containing in their sequence a base change which, when incorporated, produced a stop codon, resulting in a truncated T4 protein. This resulted in truncation of the T4 molecules at amino acid #183. An oligonucleotide comprising the sequence 5' G-AAG-GCC-TAA-AGC-ATA-G was synthesized. The normal T4 sequence is G-AAG-GCC-TCC-AGC-ATA-G. Thus, the serine encoded by TCC was mutated to a stop codon TAA and the mutant T4 protein terminated at this point.

The mutant oligonucleotide was kinased and hybridized to 10 ug of the M13 T4 template. A second strand of DNA was synthesized, using the M13 T4 template and oligonucleotide primer, by the Klenow fragment of DNA polymerase in the presence of the thionucleotide dCTP~~S~~. Any unreplicated single stranded DNA was removed by filtration through a nitro-cellulose filter and the purified double-stranded DNA was nicked with the restriction enzyme NciI. NciI will not cut phosphorothioate DNA. Thus, the new strand containing dCTP~~S~~ and the mutation were not nicked. The nicked DNA was digested with exonuclease III, which digested away the nicked, non-mutant DNA strand.

The gapped DNA was repolymerized by DNA polymeraseI, in the presence of T4 DNA ligase. In this step, the mutant strand served as the template so the mutation was copied into both strands.

The resulting DNA was transformed into competent TG1 and derived plaques were grown up. Single

-80-

stranded and replicative form DNAs were isolated and the DNA was sequenced to confirm the presence of the mutation. Mutated DNA was excised from the replicative form of DNA with Xba and ligated to Xba cut vector CDM8. This vector was developed and provided by Dr. Brian Seed (Massachusetts General Hospital). CDM8 is expressed in Cos cells upon transfection. Thus, the mutant T4 proteins could be assayed after transfection into Cos cells. CDM8 containing mutant T4 cDNAs were identified by hybridization to radiolabelled T4 DNA after transformation of the vector-insert ligation into competent MC1061P3 host bacteria. The proper orientation of insert in vector was determined by restriction enzyme analysis of mini-prep DNAs. Large scale plasmid preparations were used for transfection.

For transfections,  $2-3 \times 10^6$  Cos cells were plated in 100 cm dishes in RPMI-10% FCS-1% glutamine-1% pen-strep-10 ug/ml gentamycin. 12-24 hours later, the cells were washed with RPMI and incubated for 2-2.5 hours in the presence of 4 ml DME containing 400 ug/ml DEAE-dextran and 45 ug plasmid DNA. The cells were washed with RPMI and incubated in 10 ml DME-2% FCS-1% glutamine-15 pen-strep-10 ug/ml gentamycin-120 uM chloroquine for 3 hours. The cells are washed with RPMI and incubated for 2 days in the original media.

#### EXAMPLE 4 Production of Modified Soluble Human CD4 Fragments

Methods: The 1.17Kb T4<sub>ex1</sub> fragment was excised using BamHI from pAC 373/T4<sub>ex1</sub>, blunted using the

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Klenow fragment of DNA polymerase I, ligated to XhoI linkers (New England Biolabs) and subcloned in the XhoI site of the vector CDM8. Hussey, R.E. et al., Nature, 330:487-489 (1987); Seed, et al., Proc. Natl. Acad. Sci. U.S.A., 84:3365-3369 (1987) For

05 transfection of CDM8 constructs into Cos cells, 2-3 X  $10^6$  cells are plated in 100 X 15 cm dishes in RPMI 1640 (Gibco) containing 10% fetal bovine serum (FBS). Twelve to twenty-four hours later, 45 ug of plasmid

10 DNA are added to 2.5 ml RPMI and mixed with 2.5 ml RPMI-800 ug/ml DEAE dextran, then added to the washed Cos cells. After approximately 2 hours at 37° C, the cells are washed and then incubated in RPMI

15 streptomycin, 10 ug/ml gentamycin and 150 uM chloroquine for 3 hours. The cells were incubated at 37° C for 2 days in RPMI 10% FCS. For metabolic labelling, the transfected Cos cells (2 days after transfection) and incubated for 1 hour in 5 ml RPMI

20 minus cysteine containing 10% FCS. The media is removed and the cells are incubated in RPMI minus cysteine containing 10% dialyzed FCS and 100 uCi/ml of  $^{35}$ S-cysteine for 5-6 hours at 37° C. The supernatants are removed, centrifuged at 200 g for 10

25 minutes and dialyzed vs. PBS/0.025% azide/10mM cold cysteine overnight at 4°C. For immunoprecipitation, 5 ml of the dialyzed  $^{35}$ S-cysteine labelled supernatant is precleared by a 45 minute incubation

30 Affigel-10 (Biorad) beads (about 5 mg antibody per ml beads). The precleared supernatant is then incubated with 20 ul anti-CD4 (19Thy5D7) on Affigel-10 beads for 3 hours at 4° C. The beads are washed once in 10

-82-

ml 10 mM Tris, pH 6.8/0.1% Triton X-100/0.1% SDS/0.5%  
DOC, once in  $\pm 1$  ml of the same buffer and once in 1ml  
0.1 M glycine, pH 5/0.1% Triton X-100 and then eluted  
with 35  $\mu$ l 0.1 M glycine, pH 2/0.1% Triton X0100 and  
05 neutralized with 6  $\mu$ l 1 M Tris, pH 7.6. The sample  
is run on a 0.75 or 1.5 mm 12.5%  
mini-polyacrylamide-SDS gel under non-reducing  
conditions. The gel is fixed, dried and  
autoradiographed at about 70° C from 1-7 days.

10 Immunoprecipitation with anti-CD8 was carried out as  
above except that 20  $\mu$ l anti-CD8 on Affigel-10 beads  
is used for immunoprecipitation. For  
co-precipitation with gp120 (kind gift of Dr.  
Bolognesi, Duke University), 0.5 ml of labelled  
15 supernatant is incubated with 67 ng native gp120 for  
2 hours at 37° C. Five hundred ng anti-gp120  
(Dupont) and 10  $\mu$ l rabbit anti-mouse IgG Sepharose 4B  
beads are added and rotated for 2 hours at 4° C. The  
beads are washed once in 10 ml and once in 1 ml cold  
20 PBS, eluted and the sample run in SDS-PAGE as above.

The CD4 protein (182 amino acids long) was  
created using the thionucleotide method of  
oligonucleotide site directed mutagenesis. Taylor,  
J.W. et al., Nucl. Acids Res., 13:8749-8765 (1985);  
25 Taylor, J.W. et al., Nucl. Acids Res. 13:8765-8785  
(1985); Nakayama et al., Nucl. Acids Res.  
14:9679-9698 (1986) The XhoI insert of T4<sub>ex1</sub> was  
excised from CDM8, blunted with the Klenow fragment  
of DNA polymerase I ligated to XbaI linkers (New  
30 England Biolabs) and subcloned into M13mpl2. Single  
stranded DNA was prepared as a template and  
mutagenesis was carried out according to the  
manufacturer's recommendations (Amersham). For the

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182 amino acid truncation, the oligonucleotide 5' GAAGGCCTAAAGCATAG 3' was synthesized using standard cyanoethyl phosphoramidite chemistry. The termination codon which converts the serine (TCC) at amino acid 183 to a stop codon is underlined. The presence of the mutation was confirmed by sequencing the M13mp18-T4 construct and mini preps of the replicative form of the mutation-containing DNA were prepared. The mutated insert was excised with XbaI and ligated into the XbaI site of CDM8. The presence of the mutation was then directly confirmed by sequencing the CDM8-T4 insert using the double stranded DNA as a template. Although not shown, a truncation was also created at amino acid 110 using the oligonucleotide CACCTGCTTTAGGGGAG.

#### EXAMPLE 5 Production and Analysis of CD4

##### Site-Directed Mutants

16 CD4 mutants were constructed, as described in Example 4. As shown in Table 1, 15 oligonucleotides were used, in a standard site-directed mutagenesis protocol (Example 4), to produce 16 different version of the human CD4 molecule, each containing from 1 to 4 amino acid substitutions. As a result, the amino acid residue normally present in human CD4 protein at the position indicated in Table 1 (See Figure 1) was replaced by the amino acid present in the equivalent position of the murine CD4 sequences.

Three mutants, M3, M9 and M14, evidenced altered gp120 binding ability: M3 failed to bind gp120, M9 has substantially reduced gp120 binding capacity and M14 also demonstrates reduced gp120 binding capacity.

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The amino acid substitutions made in each are as follows:

- 05 M3 amino acid 48:P changed to G  
amino acid 50:K changed to P  
amino acid 51:L changed to S
- M9 amino acid 121:P changed to S  
amino acid 122:P changed to K  
amino acid 123:G changed to V
- 10 M14 amino acid 155:G changed to D  
amino acid 156:T changed to F  
amino acid 158:T changed to N

- |               |                  |
|---------------|------------------|
| P: proline    | S: serine        |
| K: lysine     | V: valine        |
| L: leucine    | D: aspartic acid |
| 15 G: glycine | F: phenylalanine |
| T: threonine  | N: asparagine    |

#### Equivalents

- 20 Those skilled in the art will recognize or be able to ascertain, using no more than routine experimentation, many equivalents to be specific embodiments of the invention described herein. Such equivalents are intended to be encompassed within the scope of this invention.

CLAIMS

1. A soluble human CD4 fragment capable of binding to the gp120 envelope glycoprotein of human immunodeficiency virus.
- 05 2. A soluble human CD4 fragment of Claim 1 which does not interfere with the function and the proliferation of human T lymphocytes not infected with HIV, said soluble fragment including none of the transmembrane portion of CD4 or only  
10 a portion of said transmembrane region, said portion being sufficiently small that it does not prevent solubilization of said fragment.
- 15 3. A soluble human CD4 fragment which is capable of binding to the HIV gp120 envelope glycoprotein and which does not interfere with the function and the proliferation of human T lymphocytes not infected with HIV, comprising all or a portion of the amino acid sequence of Figure 1.
- 20 4. A modified soluble human CD4 fragment which is capable of binding HIV gp120 envelope protein and which does not interfere with the function and proliferation of human T lymphocytes not infected with HIV, the fragment differing from soluble human CD4 protein by a deletion from,  
25 substitution in or addition to the amino acid sequence of human CD4 protein of at least one amino acid.

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5. A modified soluble human CD4 fragment, which is capable of binding HIV gp120 and which does not interfere with the function and proliferation of human T lymphocytes not infected by HIV,  
05 consisting essentially of the first 182 amino acids of human CD4 protein, as represented in Figure 1.
6. A modified soluble human CD4 fragment which is capable of binding HIV gp120 envelope protein  
10 and which does not interfere with the function and proliferation of human T lymphocytes not infected by HIV, consisting essentially of the first 369 amino acids of human CD4 protein.
7. A modified soluble human CD4 fragment which is  
15 capable of binding HIV gp120 envelope protein and which does not interfere with the function and proliferation of human T lymphocytes not infected by HIV, consisting essentially of domain 1, 2 and partial domain 3 CD4 protein.
- 20 8. A modified soluble human CD4 fragment of Claim 7 in which the amino acid sequence of soluble CD4 protein is truncated at amino acid position 243.
9. A modified soluble human CD4 fragment which is  
25 capable of binding HIV gp120 envelope protein and which does not interfere with the function and proliferation of human T lymphocytes not infected by HIV, consisting essentially of soluble modified human CD4 protein in which: 1)



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the asparagine present at amino acid position 271 in human CD4 protein is an aspartate and the asparagine present at amino acid 300 in human CD4 protein is an aspartate; 2) the asparagine present at amino acid position 271 in human CD4 protein is an aspartate; and 3) the asparagine present at amino acid 300 in human CD4 protein is an aspartate.

10. A biologically active, modified soluble human CD4 fragment encoded by a nucleotide sequence selected from the group consisting of:
  - a. the first 549 nucleotides of Figure 1;
  - b. the first 729 nucleotides of Figure 1; and
  - c. the first 1107 nucleotides of Figure 1.
11. A modified soluble human CD4 fragment, which binds HIV gp120 envelope protein and which does not interfere with the function and proliferation of human T lymphocytes not infected by HIV, which does not include the N-linked glycosylation sites present in the human CD4 fragment whose amino acid sequence is represented in Figure 1.
12. A soluble peptide, capable of binding HIV gp120, consisting essentially of domain I and domain II of human CD4 protein.

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- 05 13. A soluble peptide, capable of binding HIV gp120, consisting essentially of 179 amino acids, said peptide having the amino acid sequence of domain I and domain II of human CD4 protein as represented in Figure 1.
14. Modified soluble human CD4 fragments having altered gp120 binding ability.
15. Modified soluble human CD4 fragments of Claim 14 having reduced gp120 binding ability.
- 10 16. Modified soluble human CD4 fragments of Claim 14 having enhanced gp120 binding ability.
17. DNA encoding a soluble human CD4 fragment which has the ability to bind HIV gp120 envelope protein.
- 15 18. DNA of Claim 17 encoding a soluble human CD4 fragment which additionally does not interfere with the function or proliferation of human T lymphocytes which are not infected by HIV.
- 20 19. DNA encoding a modified soluble human CD4 fragment which has the ability to bind HIV gp120 envelope protein.
- 25 20. DNA of Claim 19 encoding a modified soluble human CD4 fragment which additionally does not interfere with the function or proliferation of human T lymphocytes which are not infected by HIV.

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21. DNA encoding a biologically active, modified soluble human CD4 fragment which does not include the N-linked glycosylation sites present in the human CD4 fragment whose amino acid sequence is represented in Figure 1.
22. DNA encoding a modified soluble human CD4 fragment capable of binding HIV gp120 envelope protein, said fragment consisting essentially of the first 182 amino acid of human CD4 protein.
23. DNA encoding a modified soluble human CD4 fragment capable of binding HIV gp120 envelope protein, said fragment consisting essentially of the first 369 amino acids of human CD4 protein.
24. DNA encoding a modified soluble human CD4 fragment in which the amino acid sequence is truncated at amino acid position 243, resulting in a CD4 fragment consisting essentially of domain 1, 2 and partial domain 3 CD4 protein.
25. DNA selected from the group consisting of: 1) DNA encoding soluble modified human CD4 protein in which the asparagine present at amino acid position 271 in human CD4 protein is an aspartate and the asparagine present at amino acid 300 in human CD4 protein is an aspartate; 2) DNA encoding soluble modified human CD4 protein in which the asparagine present at amino acid position 271 in human CD4 protein is an

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aspartate; and 3) DNA encoding soluble modified human CD4 protein in which the asparagine present at amino acid 300 in human CD4 protein is an aspartate.

- 05    26. DNA comprising nucleotide triplets encoding human modified soluble CD4 protein, as represented in Figure 1, except that a nucleotide triplet encoding the amino acid present in the equivalent position of murine CD4 protein has been substituted at at least one nucleotide triplet site in the DNA, the triplet selected from the group consisting of:
- 10            a. the nucleotide triplet encoding the amino acid at position 48 of human CD4 protein;
- 15            b. the nucleotide triplet encoding the amino acid at position 50 of human CD4 protein;
- c. the nucleotide triplet encoding the amino acid at position 51 of human CD4 protein; d. the nucleotide triplet encoding the amino acid at position 121 of human CD4 protein;
- 20            e. the nucleotide triplet encoding the amino acid at position 122 of human CD4 protein;
- f. the nucleotide triplet encoding the amino acid at position 123 of human CD4 protein;
- 25            g. the nucleotide triplet encoding the amino acid at position 155 of human CD4 protein;
- h. the nucleotide triplet encoding the amino acid at position 156 of human CD4 protein; and
- i. the nucleotide triplet encoding the amino acid at position 158 of human CD4 protein.
- 30

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27. A method of binding the HIV gp120 envelope protein in vitro, comprising contacting a fluid containing the HIV with a modified soluble CD4 fragment capable of binding the gp120 envelope protein, under conditions appropriate for binding of the gp120 envelope protein and the fragment to occur.
28. A method of binding HIV present in a biological sample, comprising combining the biological sample with a modified soluble CD4 fragment capable of binding the gp120 envelope protein of HIV, under conditions appropriate for binding of the fragment and the gp120 envelope protein to occur.
29. A condom comprising the modified soluble human CD4 fragment of Claim 4.
30. A composition comprising a spermicide and the modified soluble human CD4 fragment of Claim 4.
31. A device for blood collecting, blood processing and/or blood storage, comprising the modified soluble human CD4 fragment of Claim 4.
32. A medical garment, comprising the modified soluble human CD4 fragment of Claim 4.

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FIGURE 1

AAGAAAGTGGTGCTGGGCAAAAAGGGGATACAGTGGAACTGACCTGTACAGCTTCCCAG 60 bp  
TTCTTTTACCACGACCCGTTTTTTCCCCTATGTACCTTGACTGGACATGTGGAAGGGTC  
k k v v l g k k g d t v e l t c t a s q 20 aa  
AAGAAGAGCATACAATTCCACTGGAAAACTCCAACCAGATAAAGATTCTGGGAAATCAG 120  
TTCTTCTCGTATGTTAAGGTGACCTTTTTGAGGTTGGTCTATTCTAAGACCCCTTAGTC  
k k s i q f h w k n s n q i k i l g n q 40  
GGCTCCTTCTTAATAAGGTCCATCCAAGCTGAATGATCGCGCTGACTCAAGAAGAAGC 180  
CCGAGGAAGAATTGATTTCAGGTAGGTTGACTTACTAGCGCGACTGAGTTCTTCTTCG  
g s f l t k g p s k l n d r a d s r r s 60  
CTTTGGGACCAAGGAACTTCCCCCTGATCATCAAGAATCTTAAGATAGAAGACTCAGAT 240  
GAAACCCCTGGTTCCCTTGAAGGGGGACTAGTAGTTCTTAGAATTCTATCTTCTGAGTCTA  
l w d q g n f p l i i k n l k i e d s d 80  
ACTTACATCTGTGAAGTGGAGGACCAGAAGGAGGAGGTGCAATTGCTAGTGTTCGGATTG 300  
TGAATGTAGACACTTCACCTCCTGGTCTTCCTCCTCCACGTTAACGATCACAAGCCTAAC  
t y i c e v e d q k e e v q l l v f g l 100  
ACTGCCAACTCTGACACCCACCTGCTTCAGGGGCAGAGCCTGACCCCTGACCTTGGAGAGC 360  
TGACGGTTGAGACTGTGGGTGGACGAAGTCCCCCTCTCGACTGGGACTGGAACCTCTCG  
t a n s d t h l l q g q s l t l t l e s 120

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FIGURE 1 (CONT'D)

CCCCCTGGTAGTAGCCCCCTCAGTGC<sup>1</sup>CAATGTAGGAGTCCAAGGGGTAAAAACATACAGGGG 420  
-----  
GGGGGACCATCATCGGGGAGTCACCTTACATCCTCAGGTTCCCCATTTTGTATGTCCCC  
p p g s s p s v <sup>1</sup> h c r s p r g k n i q g 140  
-----

GGGAAGACCCCTCTCCGTGTCTCAGCTGGAGCTCCAGGATAGTGGCACCTGGACATGCACT 480  
-----  
CCCTTCTGGGAGAGGCACAGAGTCGACCTCGAGGTCCTATCACCGTGGACCTGTACGTGA  
g k t l s v s q l e l q d s g t w t c t 160  
-----

GTCTTGCAGAACCAAGAAGGTGGAGTTCAAAATAGACATCSTGGTGCTAGCTTTCCAG 540  
-----  
CAGAACCTCTTGGTCTTCTCCACCTCAAGTTTTATCTGTAGCACCACGATCGAAGGTC  
v l <sup>1</sup> q n q k k v e f k i d i v v l a f q 100  
-----

AAGGCCTCCAGCATAGTCTATAAGAAAGAGGGGGAACAGGTGGAGTTCTCCTTCCCACTC 600  
-----  
TTCCGGAGGTCGTATCAGATATTCTTTCTCCCCCTTGTCCACCTCAAGAGGAAGGSGAG  
k a <sup>1</sup> s s i v y k k e g e q v e f s f p l 200  
-----

GCCTTTACAGTTGAAAAGCTGACGGGCAGTGGCGAGCTGTGGTGGCAGGCGGAGAGGGCT 660  
-----  
CGGAAATGTCAACTTTTCGACTGCCCGTCACCGCTCGACACCACCGTCCGCCTCTCCCGA  
a f t v e k l t g s g e l w w q a e r a 220  
-----

TCCTCCTCCAAGTCTTGGATCACCTTTGACCTGAAGAACAAGGAAGTGTCTGTAAAACGG 720  
-----  
AGGAGGAGGTTTCAGAACCTAGTGGAAACTGGACTTCTTGTTCCTTCACAGACATTTTGCC  
s s s k s w i t f d l k n k e v s v k r 240  
-----

GTTACCCAGGACCCCTAAGCTCCAGATGGGCAAGAAGCTCCCGCTCCACCTCACCTGCCCC 780  
-----  
CAATGGGTCTCTGGGATTGGAGGTCTACCCCTTCTTTCGAGGGCGAGGTGGAGTGGGACGGG  
v t <sup>1</sup> q d p k l q m g k k l p l h l t l p 260  
-----

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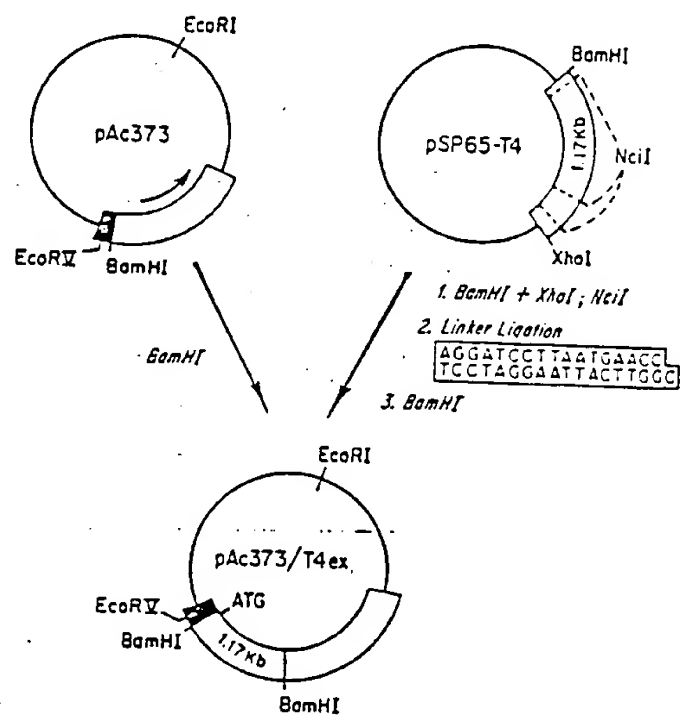
FIGURE 1 (CONT'D)

CAGGCCTTGCCTCAGTATGCTGGCTCTGGAACCTCACCCCTGGCCCTTGAAGCGAAAACA 840  
GTCCGGAACGGAGTCATACGACCGAGACCTTTGGAGTGGGACCGGGAACCTTCGCTTTTGT  
q a l p q y a g s g n l t l a l e a k t 220  
GGAAAGTTGCATCAGGAAGTGAACCTGGTGGTGTGAGAGCCACTCAGCTCCAGAAAAAT 900  
CCTTTCAACGTAGTCCTTCACTTGGACCACCACTACTCTCGGTGAGTCGAGGTCTTTTAA  
g k l h q e v n l v v m r a t q l q k n 300  
TTGACCTGTGAGGTGTGGGGACCCACCTCCCTAAGCTGATGCTGAGCTTGAAACTGGAG 960  
AACTGGACACTCCACACCCCTGGGTGGAGGGGATTGCACTACGACTCGAAGCTTTGACCTC  
l t c e v w g p t s p k l m l s l k l e 320  
AACRAGGAGGCAAAGGTCTCGAAGCGGGAGAAGGCGGTGTGGGTGCTGAACCCCTGAGGCG 1020  
TTGTTCCCTCCGTTTCCAGAGCTTCGCCCTCTTCCGCCACACCCACGACTTGGGACTCCGC  
n k e a k v s k r e k a v w v l n p e a 340  
GGGATGTGGCAGTGTCTGCTGAGTGACTCGGGACAGGTCTGCTGGAATCCAACATCAAG 1080  
CCCTACACCGTCACAGACGACTCACTGAGCCCTGTCCAGGACGACCTTAGGTTGTAGTTC  
g m w q c l l s d s g q v l l e s n i k 360  
GTTCTGCCCCACATGGTCCACCCCGGTTTCAATAA 1113  
CAAGACGGGTGTACCAGGTGGGGCCAAATAATT  
v l p t w s t p v h 370



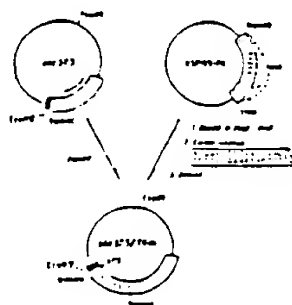
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FIGURE 2



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FIGURE 3



Excise T4 cDNA fragment  
from pAG373/T4<sub>ex</sub>

Ligate cDNA fragment  
to XbaI linkers/digest  
with XbaI

Ligate to Xba-cut M13

Introduce into competent  
TGI host bacteria

Plate/screen/select plaques  
hybridizing to sense oligo-  
nucleotides for use as  
ss M13 templates

Produce mutant oligonucleotide  
(5'-G-AAG-GCC-TAA-AGC-ATA-G)

Kinase mutant oligonucleotide  
and synthesize mutant strand  
on M13 T4 template

Produce ds DNA in which  
both strands are mutated

Excise mutated DNA from  
M13 vector with Xba

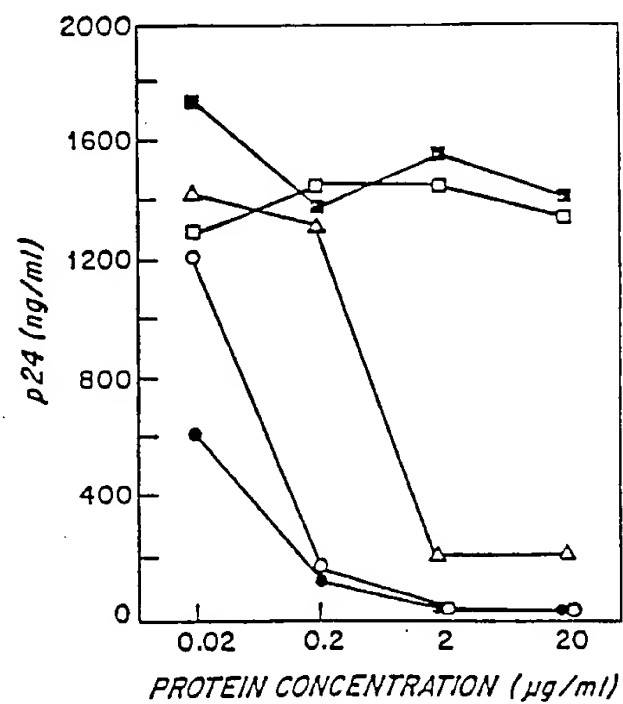
Ligate to Xba-cut CDM8

Transfect Cos cells with  
CDM8 containing mutated  
T4 DNA/express CD4 protein

Determine HIV-binding  
ability of protein  
encoded by mutated  
T4 DNA

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FIGURE 4



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FIGURE 5

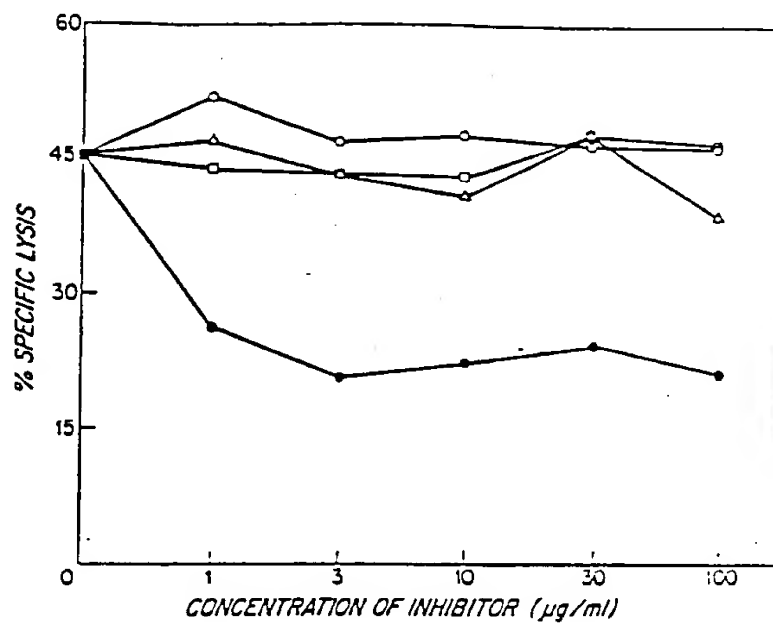
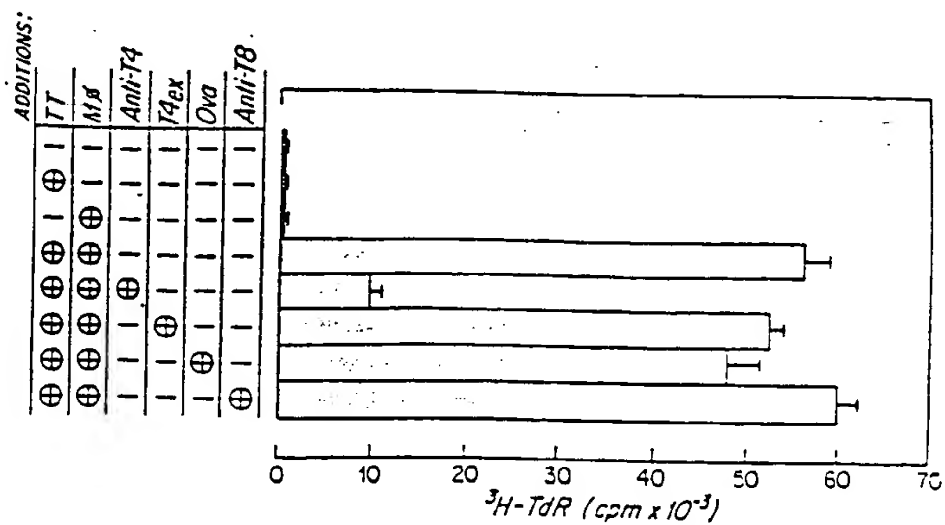


FIGURE 6



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FIGURE 7A

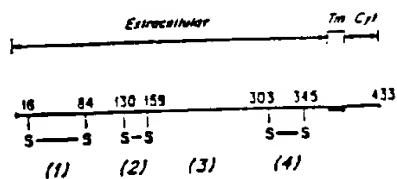


FIGURE 7B

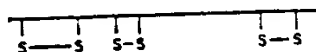
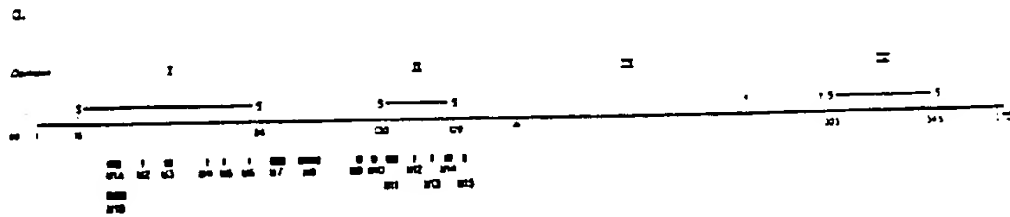


FIGURE 7C

Lys	Lys	Val	Val	Leu	Gly	Lys	Lys	Gly	Asp	Thr	Val	Glu	Leu	Thr	15
<u>523</u>	Thr	Ala	Ser	Gln	Lys	Lys	Ser	Ile	Gln	Pro	His	Trp	Lys	Asn	30
Ser	Asn	Gln	Ile	Lys	Ile	Leu	Gly	Asn	Gln	Gly	Ser	Pro	Leu	Thr	45
Lys	Gly	Pro	Ser	Lys	Leu	Asn	Asp	Arg	Ala	Asp	Ser	Arg	Arg	Ser	60
Leu	Trp	Asp	Gln	Gly	Asn	Pro	Pro	Leu	Ile	Ile	Lys	Asn	Leu	Lys	75
Ile	Glu	Asp	Ser	Asp	Thr	Tyr	Ile	<u>523</u>	Gln	Val	Glu	Asp	Gln	Lys	90
Gln	Glu	Val	Gln	Leu	Leu	Val	Pro	Gly	Leu	Thr	Ala	Asn	Ser	Asp	105
Thr	His	Leu	Leu	Gln	Gly	Gln	Ser	Leu	Thr	Leu	Thr	Leu	Glu	Ser	120
Pro	Pro	Gly	Ser	Ser	Pro	Ser	Val	Gln	<u>523</u>	Arg	Ser	Pro	Arg	Gly	135
Lys	Asn	Ile	Gln	Gly	Gly	Lys	Thr	Leu	Ser	Val	Ser	Gln	Leu	Gln	150
Leu	Gln	Asp	Ser	Gly	Thr	Trp	<u>523</u>	Thr	Val	Leu	Gln	Asn	Pro	Gln	165
Lys	Lys	Val	Glu	Pro	Lys	Ile	Asp	Ile	Val	Val	Leu	Ala	Pro	Gln	180
Lys	Ala	Ser	Ser	Ile	Val	Tyr	Lys	Lys	Glu	Gly	Gln	Val	Gln	Ser	195
Pro	Ser	Pro	Pro	Leu	Ala	Pro	Thr	Val	Glu	Lys	Leu	Thr	Gly	Ser	210
Gly	Gln	Leu	Trp	Trp	Gln	Ala	Glu	Arg	Ala	Ser	Ser	Ser	Lys	Ser	225
Trp	Ile	Thr	Pro	Asp	Leu	Lys	Asn	Lys	Gln	Val	Ser	Val	Lys	Arg	240
Val	Thr	Gln	Asp	Pro	Lys	Leu	Gln	Met	Gly	Lys	Lys	Leu	Pro	Leu	255
His	Leu	Thr	Leu	Pro	Gln	Ala	Leu	Pro	Gln	Tyr	Ala	Gly	Ser	Gly	270
<u>523</u>	Leu	Thr	Leu	Ala	Leu	Glu	Ala	Lys	Thr	Lys	Lys	Leu	His	Gln	285
Gln	Val	Asn	Leu	Val	Val	Met	Arg	Ala	Thr	Gln	Leu	Gln	Met	Leu	300
Leu	Thr	<u>523</u>	Gln	Val	Trp	Gly	Pro	Thr	Ser	Pro	Lys	Leu	Met	Leu	315
Ser	Leu	Lys	Leu	Glu	Asn	Lys	Gln	Ala	Lys	Val	Ser	Lys	Arg	<u>523</u>	330
Lys	Ala	Val	Trp	Val	Leu	Asn	Pro	Ala	Ala	Gly	Met	Trp	Gln	<u>523</u>	345
Leu	Leu	Ser	Asp	Ser	Gly	Gln	Val	Leu	Leu	Glu	Ser	Asn	Lys	Lys	360
Val	Leu	Pro	Thr	Trp	Ser	Thr	Pro	Val	His						375

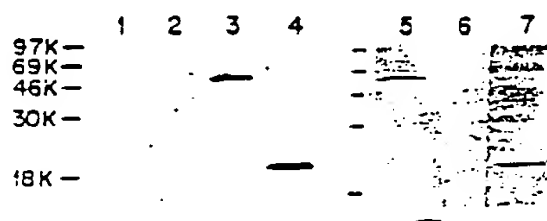
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FIGURE 8



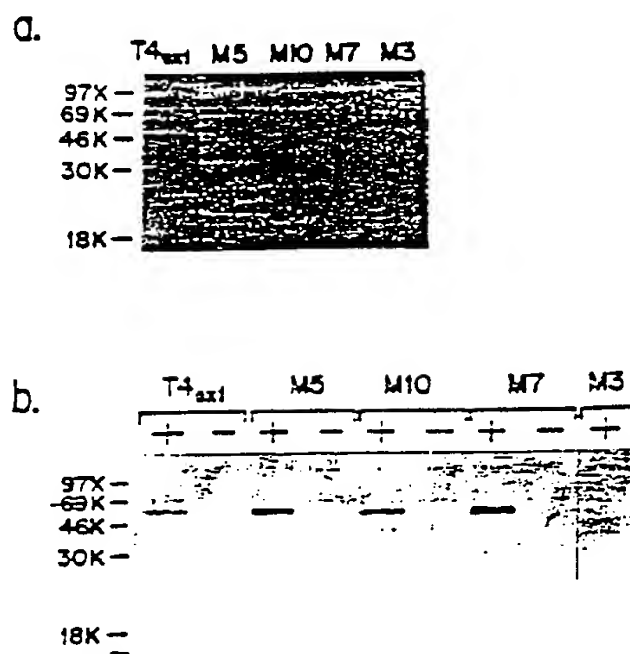
10/11

FIGURE 9



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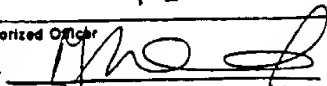
FIGURE 10





# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 88/03454

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC <sup>4</sup> : A 61 K 37/02; C 12 N 15/00; A 61 F 5/43; A 61 B 5/14		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
IPC <sup>4</sup>	A 61 K; C 12 N; C 12 P	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	Cell, volume 42, no. 1, August 1985, MIT, P.J. Maddon et al.: "The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4: a new member of the immunoglobulin gene family", pages 93-104 see figure 3, especially fragment T4B	1-26
X,P	WO, A, 88/01304 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 25 February 1988 see pages 69-75, examples 3-6	1-26
X,P	Nature, volume 331, no. 6151, 7 January 1988, (London, GB), R.E. Hussey et al.: "A soluble CD4 protein selectively inhibits HIV replication and syncytium formation", pages 78-81 see legend to figure 1 cited in the application	1-26
<p>* Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
2nd January 1989	1-2 FEB 1989	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	M. VAN MOL 	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X,P	Nature, volume 331, no. 6151, 7 January 1988, (London, GB), R.A. Fisher et al.: "HIV infection is blocked in vitro by recombinant soluble CD4", pages 76-78 see legend to figure 1  -----	1-26

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82